

Whose telephones are now free?

The British government's arrangements for regulating its newly denationalized telephone company leave a lot to be desired. The most obvious missing ingredient is competition.

JUST over a year ago, the British government sold off just under a half of the equity in its public monopoly in telecommunications, trendily called British Telecom, and it has been congratulating itself ever since on its courage and perspicacity. Certainly, the public sale of shares was the largest occasion of its kind that there has ever been. In many ways, the result has also been successful. It is, for example, now possible for British telephone subscribers legally to buy and to attach to the lines they rent from the half-private monopoly a growing variety of terminal equipment, from hand-sets to large private exchanges to facsimile transmitters. Especially in those areas of its business where independent manufacturers have competing products to offer, British Telecom has become a paragon of alertness (although it is still a time-consuming business to raise a telephone operator on a Sunday). So far, however, almost everybody who wishes to make a telephone call within Britain, or from Britain to elsewhere, must use British Telecom's telephone lines and pay whatever charges British Telecom decrees. The few exceptions are those who are already subscribers to a novel telephone network called Mercury, licensed as a separate private telecommunications network during the run-up to the sale of half of British Telecom, and which is now ironically a wholly owned subsidiary of Cable and Wireless, itself publicly-owned until three years ago. But next year, it now appears, Mercury will be a more serious thorn in British Telecom's flesh.

So much should be clear from British Telecom's howls of protest two weeks ago at the ruling on the interconnection of the two networks handed down by the body called Oftel, an independent regulatory agency set up under the Telecommunications Act to ensure that British Telecom, as a private monopoly, does not abuse its economic power. Briefly, Mercury is to be allowed to use British Telecom's network as it chooses to connect its own subscribers to the people they wish to talk to. The places at which the two trunk networks will be interconnected are specified, as are the charges that Mercury will pay to British Telecom for the use of its lines. Difficulties remain about the numbering system that Mercury will be able to use. So why was British Telecom so deeply offended that it began proclaiming that the outcome of Oftel's ruling would be higher telephone charges for domestic users of its telephone system, especially for short-distance calls? Because there is now at last a prospect that Mercury will be able to offer what British Telecom has declined to offer all these years, a service in which the prices charged for telecommunications reflect the truth about the underlying technology, that the cost of an interconnection has almost ceased to be a function of the distance between two points.

So Oftel's decision, so far as it goes, will have the welcome consequence that British telephone users will be able to communicate at prices more closely related to the real cost than has hitherto been possible. That, at least, is British Telecom's expectation, which is why its first reaction was to threaten its customers in general with higher costs for local calls. But in reality, there is no certainty that this state of affairs will come about. Mercury, like British Telecom, is in business to make a profit. Theoretically, it may believe that its business will be most efficient, and will grow most quickly, if the charges it makes for its services are somehow related to the costs of providing them, but there could easily be circumstances when it might prefer not

to grow as quickly as present opportunities suggest. For one thing, growth will entail extra capital. For another, Oftel has endorsed the British government's earlier ruling that Mercury's right to use the larger network operated by British Telecom on the terms now specified should last only for as long as Mercury has less than 7 per cent of British Telecom's business. Mercury, in other words, will not be impelled to cut prices to the bone.

This points to the weaknesses in the arrangements for competition in telecommunications attending the act of divestment. Although the British government has said that it may in due course license others to compete with British Telecom, for the time being there is only Mercury. In the United States, where the breakup of AT&T has been more radical than the sale of half of British Telecom, there is now a host of competing companies trying (and sometimes failing) to sell telecommunications more cheaply than others. If ever one should sink into a complacent relationship with the owners of the private monopoly network that it uses, another will jump in to undercut them both. The problem, in Britain, is that there is nothing to prevent such a cosy relationship between British Telecom and Mercury from growing up over the years ahead, when their public trading of insults in the past few days will have become part of folk memory. The only remedy would be to allow other companies the right to compete. The snag, as things are turning out, is that it will be technically and financially more difficult to graft later competitors onto the double-headed network now developing. Will they, like Mercury, be allowed to use the British Telecom network, and will the connections be at different places, or will they be allowed to ride piggy-back on both? The simple truth is that it would have been far simpler if the government, having decided to sell half of British Telecom, and in the cause of competition, had behaved as if it believed in competition and had allowed anybody who cared to do so to rent lines from British Telecom and make whatever use it cared to of them. As things are, the British public monopoly on telecommunications has been converted into a system of two parallel private monopolies, each of them protected by the same government. □

Tale of two systems

Why is France spending more on agricultural research while Britain is cutting back?

BRITAIN and France are both members of the European Community which is sometimes best known for its Common Agricultural Policy, an elaborate device for making Europe self-sufficient in food. As the world knows, the policy has been overabundantly successful, especially in the past few years. European farmers produce much more of almost everything than Europeans are capable of eating. Among the other consequences of this state of affairs are the high cost to European consumers of paying taxes to keep European farmers in business, the high cost of food compared with world market prices, the endless squabbles among European governments about the rights and wrongs of the agricultural policy and, latterly, transatlantic squabbling between the United States and Europe, each complaining that the other is guilty of dumping food exports at prices below production cost (which is true). But everybody



knows that this state of affairs cannot continue indefinitely. Britain, one of the chief opponents of Europe's agricultural policy, is increasingly confident that the present arrangements will one day wither away, together with the surpluses they engender. France, while pro-claiming that the policy must stand, seems also to be preparing for a change. That, at least, is one of the reasons given for the plan to reorganize the French research council INRA responsible for agricultural research (see p. 660). So why, in Britain, has the argument gone quite the other way, with the government insisting that British agricultural research, having been cut to the bone already, should be further decimated in the next two years? Sheer parsimony is not a sufficient explanation of the British policy on research (see p. 663), although it may be a large part of it. The French view appears to be that if there are to be changes in the agricultural policy, French farmers will need the benefit of new agricultural technologies if they are to survive. In Britain, the argument goes the other way. After four decades during which farming productivity has been enormously increased, and where the net cost of food imports is a smaller proportion of national wealth than for a century, Europe's surpluses are part of the reason why the government is cutting back on research. Over the three years ending in March 1988, the numbers of people employed by the chief public research organization will have fallen by nearly a third. Now the knives are out elsewhere, among the research teams of the largely successful extension service that has been one of the chief agents of increased productivity in recent decades. The French policy seems to be that farmers should be helped to greater productivity and flexibility in the face of the threatened reduction of an admittedly artificial demand for food; the British, that farmers should not be helped to be more efficient or to be able to ring the changes on the crops they grow — or that they should pay for the cost of the research that will enable them to remain competitive. The truth is that while the technology of agriculture is on the threshold of changes more radical than there have been since Neolithic times, the French line is by far the safest.

Not that the British agricultural research establishment is blameless. Over many years, and long before the Rothschild reorganization of civil science in 1971, too much of what passed for agricultural research was carried out in publicly-owned institutes dedicated to the needs of particular sectors of the agricultural industry (and sometimes, as with grasslands research, duplicated to meet the needs of different geographical regions of Britain). Too much has been done in-house, in institutes that often became centres of unchanging expertise. It was natural that agricultural research should have become the chief victim of the Rothschild review, with roughly 40 per cent of its budget transferred to the ministries concerned. But now the shoe is on the other foot. The government, in its haste to implement the policy it has just thought of, is forcing through economies at a rate that will actually increase total costs over the two financial years ahead, and before anybody can have made a sensible estimate of how a reorganized research establishment might most effectively be deployed in the interests of future farmers.

In the long run, there are good reasons why some of the costs of the research establishment should be borne by the ultimate users, the growers and the food processors. Indeed, there are many sectors of the British (or any other) agricultural industry where it would be beneficial if research could be regarded as an essential part of the production enterprise. The techniques of meristem culture are so relatively simple that no seedsman or nurseryman should be without them. And Britain might yet have an interesting cheesemaking industry if cheesemaking were not so highly centralized, and if there were bacteriologists on or near farms. The snag is that it will take time to encourage such an upheaval in the way that farmers and agricultural scientists behave. It will also take time to persuade farmers and other users of research in this field to pay for the benefits they receive, and create a mechanism for passing a hat around among them. Meanwhile, the most likely development is that the people who might perform these services will have been put out to grass. □

Curing symptoms

The US Treasury plans to cure the symptoms of the federal budget deficit. Why not the cause?

MR James Baker, the new Secretary of the US Treasury, is a new broom who is energetically doing his best to sweep clean wherever he can. Without direct influence on the legislative process, he cannot do much to cure the causes of the US money problem, the huge federal budget deficit. Three weeks ago, however, he did try to cure one of the second-order symptoms of the underlying malaise, the cry in Congress for protection from unfair competition from overseas which is a consequence of the trade deficit which is itself a consequence of the high dollar which in turn is a consequence of the need to induce overseas lenders to help bridge the budget gap. . . . The difficulty is that Mr Baker's solution, that other governments should sell their dollar holdings so as to devalue the dollar, can work only temporarily; what is to happen when other governments' dollars are all spent? Mr Baker's evident hope is that the elements of a more durable solution will be in place long before then, and he may be lucky. But nobody can be sure. And much the same sense of skating on thin ice is engendered by Mr Baker's proposals for dealing with the debts of developing countries, spelled out at the meeting of the International Monetary Fund in Seoul this month; they could work, but they might not.

The dilemma has been clear since the 1970s. Much of the more than \$350,000 million owed by the developing countries is money earned by the oil exporters when oil was still black gold, which was lent to commercial banks in the West and then passed on to developing countries so as to buy oil. (Mexico is a conspicuous exception; having plenty of its own oil, it borrowed money in anticipation of future earnings therefrom.) The same commercial banks cannot now face the reality that much of what they have lent may never be returned for fear that their balance sheets would be spattered with red ink. Even though the past three years have given banks a breathing space, the risk that excessive reality might trigger a sequence of bank failures is still present. (Those tempted to say "Serve them right!" had better reflect that the money lost would mostly belong to the banks' customers, not their shareholders, and that the results could be worldwide deflation.) So Mr Baker suggests a two-part plan; first, the commercial banks should be prepared to lend the developing countries now in debt a further substantial \$20,000 million (over the next three years) and then, that the World Bank should also lend them more.

That something needs to be done, and urgently, is plain. As things are, the risk that either the banks or their debtors will go bust is too great for comfort. And there is much in the complaints of developing countries up to their eyes in debt that far too great a slice of their export earnings is now used to pay off debts originally incurred at low interest rates but now "rescheduled" at much higher rates. The social and political consequences of that state of affairs, continued over decades ahead, are too sombre to contemplate. The flaw in Mr Baker's solution is that, like the scheme to drive down the international value of the dollar, it tackles symptoms and not causes. The total amount of debt would not shrink, but it would become less onerous in the short term. The commercial banks and the developing countries would have a breathing space but not a permanent solution to what has become their mutual problem. Nor would it help, as Mr Baker suggested at Seoul, to set up a new international bank to take over the debts on the commercial banks' books, which would be an invitation to default. The only long-term remedy is that the developing countries should be enabled more easily to pay off their debts, which in turn implies that the governments now worried about the viability of their commercial banks should be willing to provide more foreign aid to the debtor countries, and that the debtors should be helped more easily to pay off their debts by means of exports, which implies a need that interest rates should fall — and that the US deficit should shrink. □

Human growth hormone

Approval for synthetic products in US, Britain

Washington

HUMAN growth hormone for treating hypopituitary dwarfism last week became the second pharmaceutical product manufactured by recombinant DNA techniques to be approved for use in human beings. In the United States, the Food and Drug Administration (FDA) finally gave Genentech Inc. approval to market its own brand of recombinant growth hormone, called Protropin, while in Britain, the Department of Health and Social Security approved KabiVitrum's Somatom, an almost identical product manufactured under licence from Genentech.

The approvals are a coup for Genentech, which also developed the previous single recombinant human pharmaceutical, insulin, which is manufactured under licence by Eli Lilly and Co. In both countries, the product is likely to be used immediately by virtually all young people needing growth hormone supplements: use of the only alternative, growth hormone derived from human pituitary glands, was halted in May because of fears that it could be responsible for transmitting a rare and fatal viral neurological disease known as Creutzfeldt-Jakob disease (CJD).

In the United States, there have been at least three suspected cases of CJD among young adults who received human pituitary growth hormone injections as adults, and one in Britain: the disease is rare enough for this to be a suspiciously high

incidence in a small group. Intensive investigations are now under way to establish whether the pituitary product has indeed been contaminated with the infectious agent of CJD, but conclusive animal tests of infectivity take at least a year. In the meantime, it is not clear whether the suspected cases (of whom one now appears unlikely to have had the disease) presage further cases of CJD or whether those now known are likely to represent all cases caused by contaminated pituitary extracts.

Dr Albert Parlow of the University of California at Los Angeles, who has supplied pituitary products to the National Institutes of Health since 1977, claims that purification techniques he initiated then make it very unlikely that virus particles in an infected individual could still contaminate pituitary extracts; nevertheless, his laboratory is now taking extraordinary measures to eliminate virus particles, including filtration through filters with pore sizes as small as 25 nanometres.

Parlow says the ban on the use of pituitary products will represent "the genocide of pituitary research" unless FDA takes immediate steps to show convincingly that pituitary products can be made safe. Such a demonstration might be difficult: in a recent paper published in *New England Journal of Medicine*, physicians at the National Institutes of Health noted that it is prudent to assume "no amount of (chemical) processing can be guaranteed to result in a fully sterile end product".

The Genentech synthetic growth hormone is not identical with the human version, having an extra methionine residue at one end of the protein chain. Perhaps as a consequence, 30 per cent of patents develop antibodies to the product. Last year, FDA expressed concern about the immune reactions and asked for another year of follow-up data on trial patients: that period will not end until early 1986 but, doubtless impressed by the urgent need of treatment for adolescents of short stature, FDA decided after six months that its requirements had been met.

Since use of the human product was halted in May, one hundred or so hypopituitary patients in the United States who suffer from the serious complication of hypoglycaemia have been permitted to use Protropin under a special compassionate exemption; some adolescents have also been permitted to continue using human-derived growth hormone from the same production batch that they had used previously.

Genentech will soon face competition from several other manufacturers of re-

combinant growth hormone for the \$40 million US market. (The market may even grow if, as hoped, the recombinant product proves effective against other forms of dwarfism.)

In the United States, Eli Lilly has a product in clinical trials and, in Europe, Sero Laboratories is in trials in Dublin with a product manufactured by Celltech, the British biotechnology company. The Celltech product is made in mammalian cells and so lacks the troublesome extra methionine, while a Danish company, Nordisk, has found an alternative solution to the problem of producing natural sequence growth hormone.

When natural sequence products are shown to be safe and effective, they can be expected to replace the early versions, but Genentech is not planning to be left behind and is working on a natural sequence growth hormone of its own.

Tim Beardsley

Shuttle contract for Aérospatiale

Paris

HERMES, the French miniature version of the US space shuttle designed to be launched on the European rocket Ariane, is to be built under the leadership of Aérospatiale, the company that builds French nuclear missiles, it was announced last Friday (18 October).

This at last brings to an end speculation over whether Aérospatiale or the aircraft company Marcel Dassault would be prime contractor on the FF14,000 million (£1,100 million) project (see *Nature* 10 October, p.469), and will be a relief to those who suspected that a Dassault victory would compromise European collaboration on Hermes. (It was said to be pressure from Dassault that caused France to withdraw from the European advanced fighter project a few months ago.)

Dassault will not, however, go unrewarded. As makes sense, the company, partnered by another aircraft manufacturer Breugnot Aviation, will be subcontractor for the whole aeronautical system of Hermes — the components necessary for its flight in the atmosphere, as opposed to its flight in space.

The French space agency CNES which first conceived of Hermes will, alongside Aérospatiale, lead negotiations with other potential European participants, and will, according to CNES, take account of the specializations now available in the European space industry through the development of Ariane, Spacelab, and Columbus (the European contribution to the US space station). Eight countries are said to be interested in participation, but they do not include West Germany, which apparently considers its space spending on Ariane 5 and Columbus quite enough for the time being.

Robert Walgate

Soviet offer on AIDS

SOVIET doctors are prepared to cooperate with their colleagues in the United States in the fight against AIDS (acquired immune deficiency syndrome) according to Professor A. I. Vorob'ev, head of the haematological department of the Central Institute for Improving the Skills of Physicians. Writing in the newspaper *Sovetskaya Rossiya*, Vorob'ev noted that not a single case of AIDS "with the epidemiological features described by the Americans" had yet been reported in the Soviet Union.

The AIDS threat, however, is coming uncomfortably close. Poland's screening programme, launched last month (see *Nature* 12 September, p.100) has already found four instances of AIDS antibodies among the first 1,679 people screened. Only blood donors and people at special risk are being screened; the four AIDS suspects are two homosexuals (out of 52 screened) and two (out of 49) haemophiliacs who had been treated with imported clotting agents.

Vera Rich

Japanese biotechnology

Companies eye export markets

Tokyo

JAPAN'S biotechnology companies are bursting with confidence. The days when they were eagerly seeking licensing arrangements with their US and European counterparts are over. Instead, the stage may be set for international competition that will intensify in the next decade. Those seem the main conclusions to be drawn from a newly published survey of the industry.

The survey, which drew replies from 260 biotechnology companies, was carried out by the industrial newspaper *Nikkei* and its companion magazine, *Nikkei Biotech*. Almost all areas were covered, from companies manufacturing clean-rooms to those producing interferons. Expenditure on research and development appears to be doubling yearly: in 1984 it rose 28.2 per cent from the previous year, this year a 51.5 per cent rise is anticipated. Average research and development expenditure now runs at 295 million yen per company, but that disguises considerable variation.

The biotech giants, such as Ajinomoto, Toray Industries and Tanabe Seiyaku, have declared research budgets of over ¥1,000 million (US 4.6 million) a year. And other leaders, like Meiji Seika, did not give details of their expenditure. Nearly half the companies revealed that they were planning to build new research facilities, or to expand existing ones, in the near future.

The expansion is perhaps a reflection of the optimistic view of the future market for products related to biotechnology that is now emerging: from the present ¥50,000 million a year market a 170-fold increase to ¥8.3 thousand thousand million by the year 2000 was predicted by the companies surveyed.

But perhaps the most striking result of the survey is the way the gap between the United States and Japan is perceived to have shrunk. Just a couple of years ago, when *Nature* published a survey on Science in Japan, there was still considerable uncertainty over the role of biotechnology in Japan and concern over the huge lead held by the United States. The gap has not vanished in the most advanced fields. In genetic engineering, only a few companies — including Kyowa Hakko, Ajinomoto and Toray — are prepared to say they now stand shoulder-to-shoulder with US companies. None thinks it is ahead. But in cell fusion, mass cell culture and immobilized enzyme technology, which are the techniques of mass production, a handful of companies, including Meiji Seika, Sumitomo Seiyaku and Toray, now see themselves as in the lead. And in more conventional fermentation technologies, just over 10 per cent of companies saw themselves ahead of their Western coun-

terparts, and nearly 15 per cent at the same level.

The biggest remaining problems for the industry are seen to be in basic research and in the failure to obtain effective industry/university/government cooperation. These problems have been aired many times, but there are still no effective policies to deal with them. Research in biotechnology is largely funded by the companies themselves and government support of basic research remains modest. Indeed lack of support for basic research may itself become an international issue.

A great many of Japan's biotechnology researchers have trained in the United States (158 are enrolled on the National Institutes of Health visiting programme, but only six from the United Kingdom) and many are recruited direct from US universities where they are completing postdoctoral studies. Although a US

Embassy spokesman says that there is no possibility that the noble tradition of allowing other countries free access to US basic research facilities will be curtailed, complaints have already been voiced in Congress. The future may see pressure for Japan to contribute as much to the world's pool of basic research as it takes out.

Whether or not that happens, there is no doubt that there is going to be fierce competition between the United States and Japan in the next decade. And just as in the field of electronics, Japanese companies that enter the international market will have first to face fierce internal competition. It is a notable feature of the Japanese scene that many companies follow similar lines of research; one year interferon is in vogue, another year, tumour necrosis factor. Numerous companies are moving into monoclonal antibody production and the manufacture of diagnostic kits too. The companies that emerge as winners will be very tough international competitors. Biotechnology companies elsewhere should take note.

Alun Anderson

French agricultural research

New look at research council

Paris

A FRENCH dinosaur is stirring. The Institut National de la Recherche Agronomique (INRA), the national agricultural research council, which some critics say has been an agent of conservatism rather than change in French agriculture, is embracing biotechnology.

Jacques Poly, INRA director-general, has appointed Pierre Douzou, director of the national biotechnology programme, as president of INRA's scientific council. Moreover, Guy Paillotin from the Ecole Polytechnique has joined INRA as director of research, while half of all directors of INRA's laboratories have been changed in the past two years. Poly says "everything is possible now".

Not that he, or Douzou or Paillotin, has much complaint about INRA's existing research. "I'm a fan of INRA", says Douzou. INRA has world-class animal reproductive biology at Versailles, can possibly claim a world lead in classical plant embryology and has a number of major research centres as at Toulouse and Grignon, where it has set up a major effort in modern fermentation technology. But, says Poly, INRA may have been too close to the farmers, responding to their immediate needs. Now INRA must break new ground, taking account of the impact of the new biology on agriculture.

Although the shift will not be easy, "unlike your troubles in Britain", says Poly, it will not be hampered by lack of funds. INRA has not been particularly favoured under the present government, but spending will rise by 10 per cent next year. The revitalization of French agriculture through research has clearly become a

political priority. Another sign is that, in Douzou's biotechnology programme, which was reconstituted during the summer, the new "board of directors" contains only three representatives of chemical and pharmaceutical companies, but seven from the agro-food sector.

French politicians seem to have recognized that the agricultural and food industries are vast, and its second export earner. (Wine alone was worth FF18,000 million last year.) Yet these industries are threatened with fundamental change, in part because of possible adjustments to the European Community's Common Agricultural Policy (CAP) and partly from new technology in food and farming. To avoid what Douzou calls "the desertification of rural France", research, and INRA, come to the fore.

But Poly, Douzou and Paillotin recognize that INRA has been too isolated from the universities and from other research councils and that this must change. Already significant numbers of senior scientists from universities and other research councils are moving to INRA, says Poly, as a consequence of the rapid advances in molecular biology and the recent change in intellectual atmosphere in France, towards practical application.

Another key factor is the source of INRA's funds, says Poly. Since the present administration came to power in 1981, INRA has depended on the revamped and powerful ministry of research, and not on the ministry of agriculture as previously. And now the benefits are beginning to show. "It has made a big difference", says Poly.

Robert Walgate

Computer networking

IBM last come, first served again

Washington

IBM, AFTER two years of waiting and watching the office computer market, finally introduced its local area network system last week. The form of topology of the network, by which personal computers and other peripheral devices can interactively communicate, was announced in May 1984, when the company put on sale the coaxial cable that would link the devices in the network, but told customers they would have to wait two to three years for the rest of the system. Although many find IBM's new network disappointing compared with what other commercially available systems can do, the official entry of IBM into the office network market will remove much uncertainty from a notoriously slow-moving part of the computer marketplace.

The system announced last week consists of the software to link the devices and a set of chips designed by Texas Instruments that will permit the computers to be attached to the network. The Texas high-speed interface allows operations at 4 million bits per second. IBM has effectively introduced two networks, available in the first quarter of 1986, one that can link up to 72 computers on existing telephone cables and another that can connect 260 computers but requires the expensive coaxial cable.

The main complaint about the system is that IBM has not developed the technology to allow personal computers to interact intelligently with larger computers on the network. Personal computers can have access to information from mainframes, but frustrated customers must again wait until IBM introduces the interface to allow the two to interact. The company refuses to predict a date for this step.

Local area networks are not new — the most common, Ethernet, was introduced six years ago and can do much that IBM's cannot. Most existing networks use either bus or star topologies. A bus network connects peripherals to a single central cable, allowing further devices to be added easily; if one device breaks down, the system can keep on operating. The disadvantage of this system is that each component must be checked when a fault occurs; technically, the network is best suited to short distances.

A star network links peripherals through a central computer, which makes it easy to maintain but means that a breakdown of the central computer stops the whole network.

IBM has chosen to sell a network in which a series of peripherals is connected in a ring around which an electronic signal, or token, constantly circulates. A message can be attached to the token and sent to another unit in the network, which then uses the token to acknowledge the

message. No other data can be transmitted during this time. One problem with such token-ring systems is that the loss of one peripheral can close down the network; the system is also difficult to service and expand.

One complaint of Digital, IBM's competitor that offers the Ethernet system, is that established networks are standardized whereas IBM's is not. But seven major computer companies rushed to announce accommodations to their products to make them compatible with IBM's network within a day of IBM's

announcement. Such is IBM's domination of the computer market that any product it introduces becomes a *de facto* standard.

For how much longer will IBM be able to sit back and wait for others to test its markets before introducing new products? Its token-ring system may well remove some of the uncertainties and customers' nervousness from the office computer market, opening it up for customers and rival companies alike. But the long-awaited 3090 quad processor system, IBM's answer to supercomputers such as those produced by Cray and Cyber, could be a different story — customers for supercomputers are few and far between and IBM could find it has missed that particular boat.

Maxine Clarke

Information technology

Poor half-term report for Esprit

Brussels

ESPRIT, the European Commission's proud programme of collaborative research in information technology, has emerged slightly tarnished from a review of its first eighteen months of operation. The mid-term review has been carried out by a panel of independent experts whose chairman is Dr Pannenberg, chairman of the Netherlands Centre of Technology Trends and previously a vice-chairman of Philips NV.

The panel acknowledges that Esprit has helped to bring European companies and institutions together on precompetitive research, but says that the European Commission's administration of the programme has been seriously deficient. It recommends that for the remaining phases of the programme the Commission should concentrate resources on a smaller number of larger projects.

The Pannenberg review applauds the role of Esprit as a catalyst in a field in which only a quarter of Europe's needs can be met indigenously. The review also says that Esprit, as the first transnational project on such a scale in such a field, appears to be an effective mechanism for research collaboration. Saying that there has not yet been time enough to judge the success of individual projects, the review goes on to guess that Esprit by itself will not ensure an effective information technology industry in Europe, and that governments will have to pay some attention to the subsequent need for demonstration projects, prototype development and even production engineering.

The review has been based on the experience of 131 European companies working on Esprit projects, most of which seem to have found their collaborative work valuable. For the smaller companies, the 20 per cent or so increase of overhead costs entailed was considered well worth the opportunity for joint working. The development of European standards was singled out for especial praise

by many of the companies.

Most of the criticisms in the review are directed at the European Commission for having fallen down on evaluation and on the provision of communications between participants. Failures of communication hit the smallest companies and research institutes hardest, while delays in returning contracts are said to have caused cash-flow problems among the smaller companies. The review complains that the Commission's staff responsible for administering Esprit lacked industrial experience. Those responsible for evaluating applications for Esprit funds have been variously described, by participating companies, as "too academic", "lacking in knowledge" and "not always impartial".

Another common complaint is that the Esprit projects taken together do not take account of the strategic needs of a successful European industry.

The review panel recommends that the future phases of the Esprit programme should be organized in the three broad areas of hardware, software and applications and that more attention should be paid to the strategic and commercial importance of new projects.

For the second phase of the Esprit programme, the review panel wants to see a bigger budget, able to support demonstration projects as well as research. It suggests a two-tier system for the evaluation of project proposals and a more widely representative advisory board to supervise the programme. It would like to see a European network of centres of excellence as well as research fellowships.

What will happen to the panel's recommendations is far from clear. After only eighteen months, the first phase of Esprit has already eaten through most of the European Communities' contribution of 750 million ECU, originally meant to last for five years. The future will depend largely on whether the member governments are willing to find more money.

Anna Lubinska

US synchrotron radiation

Cautious start on new sources

Washington

THE US Congress has finally given the Department of Energy (DoE) the go-ahead to start pre-construction research and development for the next two major synchrotron light sources. The fiscal year 1986 appropriation for DoE, now awaiting President Reagan's signature, includes \$5 million for the design of both a 6 GeV machine providing hard X rays and a 2 GeV high brilliance machine optimized for the vacuum ultraviolet and soft X-ray regions. (The energies are those of the circulating electrons stored in the rings.)

The case for the new machines has been emphasized in several recent studies. Decisions have been spurred by the perception that Europe may be stealing a march on the United States with the possibility that a European 5–6 GeV synchrotron may be built at Grenoble (see *Nature* **315**, 362; 1985). But Congress, having recently suffered several painful experiences with major physics facilities, has said that support for pre-construction research does not imply a commitment to go ahead with construction. The 2 GeV machine is expected to cost between \$75 and \$100 million and the 6 GeV machine about twice as much. Construction would probably be financed through DoE, with the National Science Foundation (NSF) helping with planning and design.

Both machines will make maximum use of insertion devices, the value of which in increasing flux densities has been fully realized only in the past few years. Much of the initial research will concentrate on designs with several parallel insertion devices and on optics capable of handling radiation 1,000 times brighter than what is now the brightest US source of X rays and ultraviolet radiation, at the National Synchrotron Light Source at Brookhaven National Laboratory. Other existing US synchrotron light sources are at Stanford Linear Accelerator Center, Cornell University, the National Bureau of Standards in Maryland and the University of Wisconsin.

While formal decisions on construction still lie ahead, the competition to play host to one or other of the new sources has inevitably begun. Argonne National Laboratory in Illinois is making a strong pitch for the 6 GeV hard X-ray source, while Lawrence Berkeley Laboratory in California is pushing for the smaller machine. Several workshops have been held to look at design possibilities.

The most recent recommendations on synchrotron priorities were given at a briefing last month by IBM's Dean Eastman to NSF and DoE officials. Eastman confirmed the findings of his previous report on the subject a year before, conducted for the National Research Council, which gave the 6 GeV machine top priority.

But Eastman was faced with the delicate question of whether the problems experienced by the 800 MeV Aladdin machine at the University of Wisconsin — and the recent progress towards solving them — should lead to a reassessment of priorities.

Design faults in Aladdin created serious problems with beam stability, so that early this year, after 4 years of adjustments, the maximum beam current was still a paltry 2.5 mA. More recently, the incorporation of ion-clearing magnets has increased beam currents to 40 mA, and further increases are expected, but it is clear Aladdin will never approach its design current of 1 amp. Eastman was not persuaded that a major upgrade for Aladdin could substitute for the planned new 2 GeV machine.

Indeed, NSF cut off funds for Aladdin on 30 September, but David Huber, director of the Wisconsin Synchrotron Radiation Center, has nevertheless defiantly transferred beam lines from the adjacent 240 MeV Tantalus ring to Aladdin and is hoping to persuade NSF to renew its support on the grounds that even 40 mA will be useful to materials science researchers. He says that Aladdin (which, as a mark of shame, had had its name taken away by NSF) is now producing more intense light over its entire spectrum than could Tantalus. But Huber will have to persuade NSF to provide another \$2 million a year to operate Aladdin instead of Tantalus, which may not be easy, given that NSF has already once decided to wash its hands of the project.

According to Louis Ianniello, director of materials science at DoE, the experi-

ence with Aladdin "hasn't changed anything" that would affect planning for future machines. And Lewis Nosanow, his opposite number at NSF, says the design problems of synchrotrons are now largely understood — Nosanow believes that to build neither of the two proposed machines would be "a disaster", and is adamant that both are needed. Congress might decide otherwise.

Tim Beardsley

• The European Synchrotron Radiation Facility (ESRF), now firmly planned for Grenoble by a Franco-German bilateral decision, is still looking for cash. So far, France has offered to pay 39 per cent of the construction costs, West Germany 29 per cent and Italy 15 per cent. This leaves 17 per cent to find.

There have been hopes in France and Britain that a deal could be struck in which France would invest in a portion of the UK Science and Engineering Research Council's Spallation Neutron Source (SNS), which is still short of operating funds, in return for an equal investment by Britain in ESRF. But this is opposed by some leading scientists in France, who have to find users for the many neutron sources already available there. "The last thing we need is more neutrons", they say.

The ministry of research in Paris, however, is still trying to strike a deal, emphasizing that there are many experiments to be done on the pulsed SNS that could not be done anywhere else. So the saga of ESRF, first proposed by the European Science Foundation in 1973 as a facility that would give Europe a lead over the United States, continues. The news from the United States indicating that Europe could yet be overtaken will add bitterness and urgency to the story.

Robert Walgate

Wellcome aid for research

ANTICIPATING that its income will increase from about £30 million to at least £45 million next year, the Wellcome Trust hopes to spend up to £10 million annually on permanent research groups within UK universities, instead of supporting medical research only with short-term grants as it does now.

The extra income will come from the trust's sale, early next year, of one-fifth of the shares in the Wellcome Foundation, the pharmaceutical company that has been wholly owned by the trust since both were set up under the will of Sir Henry Dale. This year, the trust expects to receive about £30 million out of the profits of the foundation. Next year, it hopes earn £15–20 million in interest by investing the proceeds of the share flotation on the stock market. The problem for Dr Peter Williams, the trust's director, is how to attract sufficiently good proposals on which to spend the extra income.

It is likely, he says, that most of the

money will be spent on basic biomedical sciences, and three new advisory panels have just been set up to identify promising areas of research. But Williams is concerned that morale is so low in UK universities as to inhibit applications, and he is considering the need to attract British scientists back from the United States.

Williams is also concerned lest the increase in the Wellcome Trust's investments in UK university medical research is used as an excuse by the government to cut back on its own support. This is provided through the Medical Research Council, whose spending in universities will be matched by the trust next year.

No further sales of shares are at present planned by the trust but it may eventually sell up to 49 per cent of them. To exceed that would entail a loss of the trust's controlling interest, which would be even more contrary to the stipulations of Sir Henry Dale's will than the present sale.

Peter Newmark

British agricultural research

The worst is still to come

THE agony of the British research establishment remains where it has mostly been for several years, in agriculture. During the past few weeks, institutes of the Agricultural and Food Research Council (AFRC), such as the animal diseases institute in Berkshire and the poultry research station in Cambridgeshire, have been putting out forecasts of the numbers of people they will have to shed from the beginning of next year (35 and 26½ respectively), the Ministry of Agriculture, Fisheries and Food (MAFF) has explained how it will close four of the laboratories run by the Agriculture Development and Advisory Service (ADAS) to save £4 million a year, and the cry has gone out that the cost of applied research in agriculture must in future be borne by its beneficiaries.

The newest round of cuts is the consequence of the British government's decision, announced at the beginning of this year, that research supported by MAFF and the other agriculture ministries (in Scotland and Northern Ireland) would be reduced by £10 million in each of the years 1986–87 and 1987–88. This decision followed several years of steady decline in direct support from the government's science budget for AFRC.

The effect of the latest cuts will be to have reduced AFRC's staff by close on 2,000 in three years, or by nearly a third. During the current financial year, some 600 posts will have been lost. Some 570 posts will go next year with perhaps as many in the succeeding year.

One of the curious effects of these decisions is that the total cost of getting rid of the posts concerned is likely to exceed the money savings, at least in the short term. Paying people to retire early, paying others to move from one institute to another and the costs of reorganizing research programmes are likely to amount to £20 million in the next two years. On this occasion, however, there seems to be an understanding by the government that the burden of this "restructuring" will not fall on the science budget, but will be borne directly by central government.

Final details of the government's plans for the years ahead are likely to become known during the next few weeks. The Priorities Board, which gives advice on agricultural strategy, is soon to produce a report suggesting where cuts should fall; its guiding principle seems to be that the government should pay for research that nobody else can be expected to support. This doctrine seems to have inspired the government's present line that the beneficiaries of research should pay for it.

Later, perhaps in December, the Advisory Board for the Research Councils may have something to say in defence of AFRC in its annual advice to the Secretary of State for Education and Science.

On present form, the council may argue the need for more time if AFRC's research effort is not to be unreasonably and uneconomically reduced.

There may also be before the end of the year, a decision on the future of plant breeding research in the United Kingdom, especially that at the Plant Breeding Institute at Cambridge. For several years, the commercial value to the Treasury of the exploitation of novel strains by the Seed Development Organisation has far exceeded the cost of keeping the Plant Breeding Institute at work. Several options veering towards privatization have

been put to the government.

Further ahead, the level of agricultural spending on research beyond 1987–88 should become apparent next February, in the annual forecast of public spending for the succeeding three years. The possibility that some parts of the total cost might be shouldered by the ultimate users of research seems to vary from one field to another. On the strength of a meeting held by the Institute of Horticulture at the end of September, there seems some hope of subventions in that area, while the Milk Marketing Board and other such public organizations are potential contributors to some dairy research. But making farmers pay for research is reckoned to be possible only through legislation, for which there appears to be no preparation. □

Chemistry Nobel

New routes to X-ray phases

Washington

JEROME Karle and Herbert Hauptmann, winners of the 1985 Nobel Prize for Chemistry, developed "direct" methods of crystal structure determination while collaborating at the Naval Research Laboratory in Washington DC between the 1940s and 1960s. The award is considered by many crystallographers to be long overdue; almost all three-dimensional crystal structures are now determined using methods based on the equations derived by Karle and Hauptmann. The importance of their work, a mathematical method to measure the phases of X rays diffracted by crystals, was only generally appreciated 15 years after the first paper in a series was published in 1950.

The Hauptmann-Karle method enables phases to be calculated from structure factors, those parts of the diffracted wave that depend on the structure under investigation. To calculate the positions of atoms in a crystal, the intensities and phases of the reflections must be known. Intensities can be calculated empirically by recording the reflections on a photographic plate, where they are seen as a series of dots of different brightness. Until the Hauptmann-Karle method, the phase information was thought to be lost, and only "special molecules" (for example, those containing bromine or other large atoms) could be unambiguously resolved. Isomorphous replacement, in which heavy metal ions are bound to large molecules, gave Max Perutz and John Kendrew the Nobel prize in 1962 for determining the first three-dimensional structures of proteins, but this method is not suitable for smaller molecules.

Hauptmann and Karle, using the fact that the electron densities of atoms in a crystal can never be less than zero, developed a series of equations to describe the limitations of the possibilities of phase displacements of diffracted waves. The practical applications of these equations

became apparent when David Sayre used a more intuitive approach to derive the same relationships, and Isabel Karle developed a symbolic addition procedure. The first application allowed the structure of a 15-atom antibiotic molecule to be calculated in two years. Computer programs based on the equations, originally developed by M. Woolfson and colleagues at the University of York, now allow the structure of a 50-atom molecule to be determined in two days.

The next challenge will be to apply the method to larger molecules such as proteins; Karle and Hauptmann are now trying to do exactly that. **Maxine Clarke**

Astronomer's prize

THE US astrophysicist Lyman Spitzer Jr has received the Royal Swedish Academy of Sciences' first Crafoord prize for astronomy. The prize, worth US\$135,000, was awarded for "fundamental pioneering studies of practically every aspect of the interstellar medium, culminating in the results obtained using the Copernicus satellite".

Since the 1940s, Lyman Spitzer has explored all aspects of the physics of the interstellar medium, especially in the ultraviolet. Following pioneering work with balloon-borne telescopes, he led the work on the third of the National Aeronautics and Space Administration (NASA)'s Orbiting Astronomical Observatories, which became known as the Copernicus satellite. One of the discoveries made with this satellite was that our Galaxy is surrounded by a corona of very hot million-degree gas, the existence of which Spitzer had predicted on theoretical grounds. He also had a major hand in the acceptance of the Hubble Space Telescope by NASA.

The Crafoord prize is awarded alternately for mathematics, geoscience, bioscience and astronomy and was first awarded in 1982.

M. Rowan-Robinson

Eureka

Brussels not to be the fulcrum

Brussels

A MEETING in Bonn last week put the finishing touches to the charter for Eureka, the French-inspired plan to stimulate European collaboration in advanced technology. The charter is the basis on which European foreign and research ministers will formally launch Eureka at a meeting at Hannover on 5 and 6 November.

What emerges from the charter is a flexible administrative structure under which it would be up to European companies to work out among themselves projects on which they might collaborate and then to seek government approval. Companies will usually have to foot whatever bills arise from their own resources.

Under the charter, participating governments (which could include governments not members of the European Community) will appoint a "high representative" as the focus for plans drawn up by national companies and also a means of liaison with opposite numbers elsewhere. There will be regular meetings of research ministers from participating countries, with a rotating chairmanship.

The role foreseen for the European Commission is not the dominant one for which some in Brussels had been hoping. The Commission will participate in Eureka as if it were a government, but the

charter foresees scope for "supportive measures" that the Commission may be able to provide.

At next month's meeting in Hannover, representatives will be asked to consider a number of specific projects for European collaboration, including the development of a 64-megabit memory chip, micro-processors with operating cycle times measured in picoseconds and high-power industrial lasers.

Meanwhile, it seems that the West German government is backing away from its earlier offer to make extra funds available for Eureka projects. The finance minister, Gerhard Stoltenberg, has now made it plain that any such funds would have to come from the 1986 research budget (DM7,500 million for next year). But there are reports that West German research minister Heinz Riesenhuber has not yet abandoned hope of raising DM1,000 million to support Eureka during 1987-88.

Elsewhere, France has committed FF1,000 million for Eureka but it is not clear how much of the money is new, or simply relabelled. The German view is close to the British government, which is insisting that Eureka projects should be supported either by the companies involved or by the usual sources of venture capital.

Anna Lubinska

Eureka

Avoiding the Esprit pitfalls

Paris

THE French view of European Commission participation in their pet project Eureka, originally inspired by the challenge of the US Strategic Defense Initiative (SDI or "star wars"), is that the Commission should enter only as another nation state, and not as an overall controlling body. And now the French view seems to have prevailed (see above).

Last week senior advisers of the French research minister, Hubert Curien, explained their motives. "We had to avoid the sirens of Esprit", the European research programme in information technology established by Belgian Commissioner Etienne Davignon 2-3 years ago, they said. It seems the French have bad memories of the early days of Esprit. The Commission apparently proposed a bureaucracy of some hundreds of officials to run it, and both Britain and France balked at the prospect. After a struggle, the two countries established a "light" management structure, under which Esprit has had some "positive" results.

It is just because of this success that the risk was great that Eureka would be drawn inexorably into the Brussels net, where it would not flourish. Eureka will have to

have *à la carte* project financing, partners outside the European Community, and is designed to produce commercially competitive projects, none of which is easy under the Treaty of Rome. Eureka must also be close to and highly responsive to industry. Esprit had shown some of these qualities, but it involved only pre-competitive research. However successful Esprit had been (for the latest assessment see page 661), the commercial qualities needed of Eureka put it in another class.

As for the structure now proposed, in which nations will be able to play their own cards more vigorously than within the European Council of Ministers, this would not exaggerate national tensions and divergences they say in Paris. Rather each state brings its own views — leading to "extraordinarily rich" debate. Even the British view that Eureka must be market-led, in contrast to the French technology-pull approach, is a welcome contribution.

But France is not intending to "short-circuit" the Community, Curien's advisers insisted. Eureka needs a structure that derives from industry and is not imposed from above. This is an action in the Community's interests, Paris officials say.

Robert Walgate

A germ of an idea

Paris

"ARTIFICIAL SEEDS" are at the top of the French proposals in biotechnology for the Eureka high-technology collaboration programme, due to be discussed at ministerial level in Hannover on 5-6 November. According to Yves Demarly, the Orsay plant scientist who will lead the programme if it gains European support, the idea is to coat minute somatic plant embryos — embryos derived from cell cultures rather than seeds — with some protective substance so that they could be distributed just like seeds themselves.

The advantage is that it would be possible to distribute clones of precise genetic content, as opposed to the relatively random genes of seeds, and to prepare hybrids that could not be obtained classically by sexual recombination.

The embryos would have to be stabilized (at present they would last only a few days) and the coats would have to be designed to dissolve or otherwise disintegrate in the field. The coats could contain a food supply, systems to protect the embryo against pathogens in the soil (such as antagonistic bacteria or fungi), and "starter substances" — plant hormones such as auxins and cytokinins.

"We can already coat somatic embryos of lucerne [a legume] in a kind of jelly, and get 100 per cent germination", Demarly



says, but there is a long way to go before the concept could be made practical. But patents are already being taken out on artificial coatings in the United States, and France is beginning to see the danger to its seed companies, which have an extraordinary 10 per cent of the £20,000 million world seed market. Eureka's role would be to establish and support a European development programme in the area.

Already 30 companies have shown an interest in the project, including France's largest chemicals company Rhône-Poulenc, the principal French seed company Limagrain and the Swiss pharmaceuticals giant Ciba-Geigy. With so many interested parties, it will be difficult to settle on a definite proposal in time for Hannover.

Robert Walgate

Leprosy vaccine

Large-scale trials begin in India

New Delhi

THE first large-scale clinical trial of the anti-leprosy vaccine derived from armadillos will begin two months from now in India. This follows closely on the announcement of a similar trial in Malawi (see *Nature* 316, 183; 1985). The vaccine has been developed under the aegis of the World Health Organization (WHO), but the Indian trial is being mounted by the Indian Council for Medical Research (ICMR). This international project will thus take place soon after the clinical trial of the Indian anti-leprosy vaccine developed at the Cancer Research Institute.

The trial of the WHO vaccine will be mounted, once the Drug Controller of India has given formal clearance, in the Chingleput district of Tamil Nadu in south India. Both leprosy and tuberculosis are endemic among the 4 million people living there. The director-general of ICMR, Dr V. Ramalingaswami, says that the council's Jalma Institute of Leprosy will be responsible for planning and carrying out the trial, and that it is hoped to vaccinate up to 300,000 apparently healthy contacts of leprosy patients.

The first objective is to assess the prophylactic efficacy of the vaccine, by comparing the vaccinated group with those among the population who are not vaccinated. There will also be a comparison of the value of the WHO vaccine with and without accompanying Bacille Calmette-Guérin (BCG). The groups involved will be followed for ten years.

Ramalingaswami says that ICMR has agreed to test the vaccine only on condition that WHO will also supply the technology for manufacturing the vaccine locally. What this means is not clear, for ICMR does not have facilities for maintaining an armadillo colony. All but two of

the six animals imported by the Jalma Institute of Leprosy four years ago have since died. It is believed that ICMR will import the *Mycobacterium leprae* harvested from armadillos and set up processing facilities in India using the WHO technology. Meanwhile, vaccine for the Chingleput trial will be supplied by Burroughs Wellcome of Britain.

This may be the first time that India has insisted on technology transfer as a condition for testing a WHO product. In the past, many in India have complained that multinationals have tested their products under WHO auspices and have then exploited the Indian market without offering price concessions or transferring, on easy terms, know-how for manufacturing the products. For the leprosy trial, ICMR has been under pressure to test the WHO vaccine and had to choose between that and an Indian vaccine which has already completed phase-I trial and been given clear-

ance from the toxicology angle by the Drug Controller.

Ramalingaswami said ICMR had decided to try both vaccines. A trial of the vaccine developed by the Cancer Research Institute (CRI) in Bombay was begun on 2 October (Mahatma Gandhi's birthday) at Wardha in central India. This vaccine is made from gamma-ray-inactivated cultivable mycobacterium (belonging to the *M. avium* group) isolated from human leproma and showing extensive cross-reactivity with *M. leprae*. The number of people to be vaccinated in Wardha will be the same as in Chingleput. But the CRI vaccine will be given to leprosy patients as well as to contacts, for the phase I trial showed therapeutic potential as well as prophylactic effect.

India has four million leprosy cases — one-third of the world's total — and there are 300,000 new cases a year. ICMR says it will be several years before the results of the two trials are known, so chemotherapy will continue to be the main plank for achieving India's goal of eradicating leprosy by the year 2000. **K.S. Jayaraman**

US standards bureau

Emergency measures required

Washington

A CONGRESSIONAL committee has heard dire warnings that the national measurement system could face "total breakdown" unless urgent steps are taken to reverse the decline of the National Bureau of Standards (NBS). Edward Nemeroff, president of Datron Instruments, told the House of Representatives' science, research and technology subcommittee that many users of NBS's calibration services were unable to meet the needs of their organizations because NBS could not provide necessary measurement standards and services.

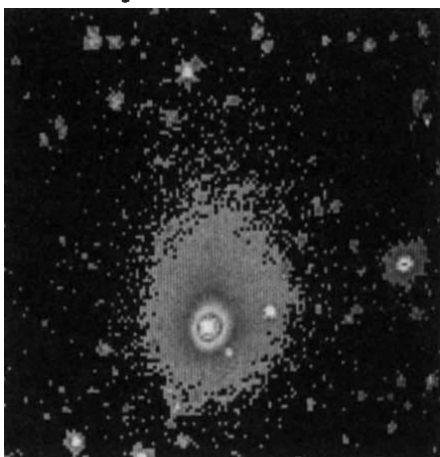
To be fair to NBS, other witnesses called to the special two-day hearing earlier this month on the future of NBS had kinder things to say about the bureau's work of developing and providing measurement standards for industry in fields as different as microelectronics and fire safety. But the hearing provided a forum for growing concern that the Reagan administration's perennial attempts to cut NBS's budget (always partly restored by Congress) could seriously increase measurement costs for industry. Despite attempts in Congress earlier this year to add \$20 million to the administration's proposed \$120 million appropriation for NBS in fiscal year 1986 (which started 1 October), the figure is likely to end up at no more than the authorized \$124.5 million — a decrease in real terms, since the appropriation has hovered at \$120 million for the past four years. The number of staff employed at NBS — of whom a high proportion are scientists — has fallen by 16 per cent since 1980.

Behind the unrest is the fact that NBS has sought to keep up with advances in technology within a shrinking budget by cutting back in more pedestrian areas. High priority targets for expansion include supercomputers; high performance ceramics (which will be shortly aided by a new cold neutron guide hall); optical electronics; and biotechnology. In order to finance these new activities, consumer product measurements have been reduced and the administration has for each of the past four years proposed closing NBS's building technology and fire research laboratories, so far without success. Even NBS's director, Ernest Ambler, admitted that "we've got our backs to the wall".

NBS is currently administered by the Department of Commerce. One proposal that has surfaced many times in the past is that NBS should be found a more suitable home, perhaps with the National Science Foundation in a new Department of Science and Technology. But Ambler shows little enthusiasm for the idea, and the Commerce Department is thought unlikely to want to surrender NBS, which accounts for a substantial fraction of its budget. Without President Reagan's personal support for the plan it now appears unlikely to go ahead. And that, according to congressional staff, means there is no easy end in sight for NBS's tribulations: because it is classified with the Commerce Department, NBS is denied the influential support of the champions of scientific research and development in the Office of Management and Budget who have successfully defended the science budget in the past.

Tim Beardsley

Halley's Comet



The forming tail of Halley's Comet, as seen from the 60-inch telescope at Palomar Observatory on 25 September.

New Zealand research

SIR—My vain wait for responses to your recent survey of science in Australasia (*Nature* 316, 185; 1985) from more senior New Zealand academics than I suggests that the "philosophical resignation" perceived amongst scientists in the New Zealand university research community is widespread. Alternatively, the 37 per cent salary raise for senior staff announced last week has pacified those who might be "furious about their current situation". Whatever the reasons for the silence in spite of the printed inaccuracies in research and development funding which have since been corrected (*Nature* 316, 668; 1985), I wish to correct two other errors and add my own pennyworth on the vexed subject of research funding in New Zealand universities.

First, the daily subsistence for New Zealand academics on sabbatical leave was reported to be NZ\$20, when in fact at this university it is NZ\$50. Even so, the maximum single leave allowance equivalent to £2,050 is inadequate.

Next, one government research body was conspicuously absent from the schematic representation of New Zealand's scientific hierarchy — the Wildlife Service of the Department of International Affairs. Although only a dozen scientists are involved, they have rescued critically endangered avian species from the brink of extinction and are highly respected in this country. As of April 1986, the Wildlife Service and several other departmental groups of biologists will be re-deployed in a new Conservation Department.

This recently announced move has been debated for months and has important ramifications for future ecological research in this country. Although *Nature's* survey was "necessarily incomplete", the lack of any reference to a proposal to amalgamate 800 scientific and support staff from at least four existing departments into one unit with a conservation mandate surprised me.

Finally, I reject the idea that the too-even distributions of funds which prohibits New Zealand universities from, for example, buying large items of equipment, is the fault of the "cosy three wise men" from the University Grants Committee who review applications for financial support, and that the cure lies in a "stiff dose of competitive peer review". The implied paternalism is nonexistent in my short experience.

The real problem lies in the distribution of resources within a university, rather than between them. Annual requests for equipment funding are ranked by the heads of the departments, and then within the university by a subcommittee of the Research Committee, according to unknown criteria. The paradox is that while a behavioural ecologist like myself can

readily obtain modest funds for gear but not for field assistance or vehicle expenses, laboratory researchers seemingly enjoy a superfluity of technicians but are unable to purchase expensive machines with which to occupy them! Even when I sought "contentment in an external source of funds" amounting in total to four times the 1984 maintenance grant for this department, it was impossible to judge whether the nebulous criteria for promotion had been satisfied and in the absence of peer review, whether anything other than my age relative to my salary was considered.

The isolation of New Zealand from the rest of the world so eloquently illustrated on your cover (*Nature* 18 July) is not the most immediate problem facing university researchers here.

CLARE VELTMAN

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SCOPE response

SIR—Your issue of 19 September (p.189) gave accounts of the SCOPE report made in Washington on the environmental effects of nuclear war. Last year (*Nature* 312, 696; 1984) you criticized SCOPE for discussing moral issues with religious leaders in Bellagio. Your editorial now criticizes our lack of courage in facing the political implications. They were not in our terms of reference and I could not have brought this technical study to an end in two years if policy had to be discussed with a steering committee of French, Swedish, Dutch, Indian, Russian, Japanese, American and British representatives of their national academies and with the 300 scientists who volunteered to work.

To come to conclusions on climatic effects, we concentrated on smoke and attempted to make estimates independent of scenarios on nuclear exchange. In this way, 100 major cities burnt would give enough smoke and require only a limited number of weapons in a counter-value exchange, not the 6,000 MT yield from total exchange of 13,000 MT in the arsenals. The figure for yield had to be used for the new fallout and unshielded dose calculations which revise those Glasstone and Dolan made in 1977.

We did comment on small exchanges in discussing the effect of single bursts at 25–50 km altitude on communications through the electromagnetic pulse (EMP), and the volume on Biological and Agricultural Effects avoids reliance on major exchange scenarios by concentrating on small stresses.

Should we have spelled out more clearly that a nuclear attack on major cities and

industrial installations could rebound on the attacker and non-combatants? When the report is published later this year, and is translated into Russian, Japanese, Chinese, French and possibly German, it may be used in many ways to support different standpoints. We had hoped by holding workshops in Sweden, India, England, Soviet Union (including Estonia), Italy, the Netherlands, France, Japan, Canada, Venezuela, Australia and the United States (and you have credited SCOPE with openness in this) that one-sided use would be minimized.

FREDERICK WARNER
(Chairman, SCOPE)

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PhD theses

SIR—I agree with Beverley Halstead (*Nature* 29 August, p. 763) that the PhD examination needs to be reformed. Having examined more than 50 candidates over many years, I have found theses get longer and more material remains unpublished.

Twenty years ago I wrote several articles (in *Biologist* and elsewhere) suggesting that the thesis in its existing form be abolished and that candidates should submit published work or (where gaps in results still existed) in the form required for a named journal. This would at least teach the candidate to read the instructions of the journal, something that many scientists of standing still do not do.

Students need to learn to use the literature. Much of most theses consists of material dragged in from the margins of the subject, to impress the examiner. I suggested that a separate literature review, on a topic wider than the research, on the lines of *Biological Reviews*, might be useful.

For what it is worth, my own PhD thesis consisted simply of a reprint of a 14-page paper published in the *Proceedings of the Royal Society*.

KENNETH MELLANBY

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Avoiding AIDS

SIR—Your editorial on AIDS (acquired immune deficiency syndrome) and the public anxiety about it assiduously avoids the main point.

Aside from the risk associated with being given certain blood products, if you are not promiscuous, you will not get AIDS.

Has chastity replaced death as the great unmentionable?

IAN DAVIDSON

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Electron gases make their mark

This year's Nobel Prize for Physics is awarded to Dr Klaus von Klitzing for his work on the quantum Hall effect. The development is an important pointer to a field of physics still poorly understood.

PROFESSOR Klaus von Klitzing, director of the Max Planck institute of solid state physics at Stuttgart, said earlier this week that he hoped it would not be another 22 years before a West German won a Nobel Prize. Modestly, he was referring to the prize awarded that long ago to the Munich physicist Mossbauer for the discovery that still bears his name, and to the substantial investment in basic science by the West German government over recent decades. Most observers expect von Klitzing's hope to be fulfilled.

As with Mossbauer's work, the importance of von Klitzing's research has been quickly recognized. His first published article on what is now called the quantized Hall effect (with G. Dorda of the Siemens research laboratory at Munich and M. Pepper of the Cavendish Laboratory, Cambridge) appeared only in 1980 (*Phys. Rev. Lett.* **45**, 494; 1980). The objective then was to devise an accurate determination of the fine-structure constant (h/e^2 , where h is Planck's constant and e the electric charge of the electron) in terms of a standard of electrical resistance. As things are turning out, it seems just as likely that von Klitzing's original experiment may be used the other way around, to provide convenient and portable standards of electrical resistance that can be directly related to fundamental constants.

The essential novelty of the quantum Hall effect is that it is one of the complicated series of phenomena observed in the now fashionable "two-dimensional electron gases", the planar layers of essentially free electrons that are inferred to exist most simply at the boundaries between, say, the semiconductors GaAs and GaAlAs when these are grown together epitaxially to form a heterostructure. They also occur in an oxide layer sandwiched between a metal and a semiconductor.

These two-dimensional systems are now all the rage. The population of electrons in the transition plane can be determined at will by suitably biasing the two poles of the device. The particular interest of von Klitzing's work is that it exploits the way in which, in strong magnetic fields perpendicular to the plane of the two-dimensional electron gas, the cyclotron motion of the electrons is quantized; only specified states of motion are allowed.

The analogy with the classical Hall effect is skin-deep only. That phenomenon is most simply that by which a

magnetic field perpendicular to an electrical current flowing in a conductor will excite an electrical voltage in the direction at right angles to the field and to the current. Most simply, the Hall effect is a direct prediction of Maxwell's equations: alternatively, in baby-talk, it is a reflection of the tendency for electrons moving at right angles to a magnetic field to be deflected into cyclotron orbits which, being disallowed, shows up as an electrical potential across the conductor.

For practical purposes, the Hall voltage is classically proportional to the external magnetic field. The phenomenon is a convenient way of studying the properties of electrons in metals and semiconductors. It is, for example the standard way of determining electron mobility. Given the general ambition now to make a practical device of more or less any physical phenomenon, the classical Hall effect is potentially a means by which external magnetic fields might be used (without physical contacts) for generating transverse voltages in current-carrying conductors so as to make novel kinds of switches for applications not yet identified.

The quantum analogue of the classical Hall effect owes much to the work of the Soviet physicist Landau, who was the first to show that the motion of electrons constrained to move in a plane would be quantized in the presence of a perpendicular magnetic field. This leads to the conclusion that the Hall conductance (the ratio of current to transverse voltage) should itself be a multiple of the fine structure constant. (The simple expectation is that the Hall conductivity should be an integral multiple of the inverse of the fine-structure constant.)

Making these phenomena susceptible to observation has been a technical triumph of the first order. Liquid helium temperatures are unavoidable. Very high magnetic fields (20 tesla or more) are usually necessary but, given the need for liquid helium anyway, may conveniently be provided by superconductors.

Von Klitzing's original measurement of the fine-structure constant in terms of a known resistance, repeated many times since 1980, has now been shown to be capable of an accuracy of a few parts in 10,000 million, although the present state of the measurements is limited by the accuracy with which the fundamental constants are known. But the availability of a system of measurement of such potential

precision, greater even than that so far demonstrated for Josephson junctions, will hasten the day when it becomes prudent to fix the value of some composite constant, such as the fine-structure constant, and to allow the unit of electrical resistance to be fixed in terms only of atomic quantities such as these.

This is the promise offered when the quantum Hall effect was in its infancy. Since then, a great many surprises have made the field even more intriguing. Not the least has been the discovery that the Landau levels are not always simply quantized, but that the quantum numbers may be fractional rather than integral (or that a two-dimensional electron gas at low temperatures may behave as if it were made of particles with fractional electrical charge).

Nobody seriously suggests that physics is being turned on its head in ways like these. Rather, the inference is that the fractional quantization of the two-dimensional electron gas is a measure of collective motions that are themselves quantized. In other words, the systems behave as if they were two-dimensional quantum liquids — whence the discovery that, under sufficiently extreme conditions, the liquid may be crystallized.

For the immediate future, the absorbing interest is to find a better understanding of the behaviour of these previously unknown electronic structures. The theoreticians have much to do, but the recognition that there are close similarities with at least some models of superconductivity is obviously a challenge. Meanwhile, the experimentalists are doing what they can to determine the structure of electronic states in these two-dimensional structures.

Ultimately, no doubt, there will be devices as well. Perhaps the most hopeful direction in which to look for these is that the population of electrons in the two-dimensional layer can so easily be altered by suitable adjustment of the electrical bias of the system, suggesting means by which conduction in the plane of the layer may be disproportionately affected. But despite the tradition that a phenomenon that does not lend itself to the construction of a laser is almost certain to make a transistor of some kind, these are early days for that train of speculation. What von Klitzing has done is to open up a whole stack of phenomena whose explanations are still incomplete.

John Maddox

Plant breeding

Engineering herbicide resistance

from Robert Shields

ONE of the much-vaunted targets for genetic engineering in agriculture has been the production of herbicide-resistant plants. To be useful, herbicides must be selective, that is they must kill plants but not animals, and weeds but not the crop. There are many problems in achieving the second objective, so it is not surprising that several agrochemical companies are looking to genetic engineering to produce herbicide-resistant crop plants. On page 741 of this issue¹, Comai *et al.* show that this prospect is not just an idle dream.

It is relatively easy to produce herbicides selective for plants. Many in current use affect functions unique to plants: atrazine and diuron interfere with photosynthesis, and glyphosate, the sulphonylureas and imidazolinones block the synthesis of essential amino acids. But crop plants share these processes with weeds, so crop plants must be protected in other ways, for example through differential uptake and metabolism of the herbicide by the crop and weed or by careful choice of the time and site of herbicide application. The differential sensitivity of plants to herbicides can cause problems. For instance, atrazine is an effective herbicide for use with maize, which can detoxify the compound, but residues remaining in the soil cause problems when maize is rotated with the more sensitive soybean. If soybean could be made atrazine resistant, the problem would be avoided. Other broader spectrum herbicides such as glyphosate are rapidly broken down by soil microorganisms, so soil residues are less of a problem, but glyphosate is indiscriminate in the plant attacked. If selected plants could be made glyphosate resistant then this herbicide could have wider applications — and higher sales.

Comai and colleagues started from the observation that the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), an enzyme involved in aromatic amino acid synthesis, is the *in vivo* target for glyphosate in bacteria, and then selected glyphosate-resistant strains of *Salmonella typhimurium*. Some of these bacteria contained the gene coding for a resistant version of EPSP synthase. The mutant form of the enzyme differs from the wild type by a single amino acid. The coding region of the resistant bacterial EPSP synthase was hooked up to eukaryotic promoter and poly (A) addition sequences derived from the T regions of agrobacteria and the resulting chimaeric constructs recombined into the T₁ region of *Agrobacterium rhizogenes*, which is the causative agent of 'hairy root' disease. Like the better studied tumour-forming species *A. tumefaciens*, to which it is re-

lated, *A. rhizogenes* contains a large plasmid. The transferred (T) region of this root-inducing (Ri) plasmid contains genes with eukaryotic transcription signals and is transferred to the plant, where it is stably incorporated in nuclear DNA. The strain of *A. rhizogenes* used in these experiments has two T DNA regions in its Ri plasmid (termed T_L and T_R), which are independently transferred to the plant. Genes in both the T_L and T_R region promote root formation in transformed plant tissue; genes in the T_L region are responsible for the extreme hairy phenotype. The advantage of *A. rhizogenes* over *A. tumefaciens* is that it is relatively easy to regenerate plants from 'hairy roots', whereas plants can only be regenerated from *A. tumefaciens*-transformed tissues if the tumour genes have previously been mutated or deleted.

In the experiments reported by Comai *et al.*, a strain of *A. rhizogenes* carrying the chimaeric EPSP synthase in their Ri plasmids was used to infect tobacco leaf disks and the resulting roots were regenerated into plants. The resistant EPSP gene inserted into the plant genome was correctly transcribed and translated, so that the regenerated plants were resistant to glyphosate, with the degree of tolerance depending on the level of EPSP synthase expression. So these experiments show that it is indeed possible to transfer herbicide resistance into a previously sensitive plant.

One intriguing aspect of these results is that plant amino-acid biosynthesis is conducted by enzymes encoded by nuclear genes that function in the chloroplast, whereas the transferred resistant EPSP synthase works in the cytoplasm. Presumably the chloroplast is permeable to the precursors and products of the synthase. It would be interesting to know if higher levels of herbicide tolerance could be achieved if the resistant enzyme were directed into the chloroplast where the sensitive plant enzyme resides.

Recent experiments have shown that it is indeed possible to transfer foreign proteins encoded in the nucleus to chloroplasts if the proteins are linked to chloroplast-specific transit peptides^{2,3}. In these experiments the bacterial enzyme, neomycin phosphotransferase (npt-II), was transferred to chloroplasts of plants transformed by *Agrobacterium* vectors carrying the *nptII* gene in a chimaeric construct with the transit sequence from the gene for the small subunit of ribulose biphosphate carboxylase. These experiments are of great practical importance for the engineering of herbicide resistant crops. Several herbicides besides glypho-

sate block chloroplast-located amino acid biosynthesis; for instance, the very broad spectrum sulphonylurea herbicides interfere with acetolactate synthase (ALS), the first enzyme in the biosynthetic pathway of branched-chain amino acids. Genes coding for a resistant version of ALS have been located in yeasts and it should now be a relatively straightforward matter to transfer them to plants.

A bigger challenge for the genetic engineers is provided by the herbicides such as atrazine and the chemically unrelated diuron that interfere with the binding of plastoquinone to the Q_B protein, part of the photosystem II complex. The Q_B protein is encoded by chloroplast DNA and is firmly anchored in the thylakoid membrane of that organelle. Plants and a number of microorganisms have been found that are resistant to atrazine, and a recent report describes the cloning of a gene coding for diuron-resistant Q_B protein from a cyanobacterium⁴. The atrazine- and diuron-resistant Q_B proteins from a number of sources differ from the wild type by a single amino-acid substitution at the same place in the protein chain. Would it be possible to engineer atrazine and diuron resistance in plants by expressing chimaeric genes encoded in the nucleus that code for a mutant Q_B protein linked to a chloroplast transit peptide? Problems could arise with such a strategy because the extremely hydrophobic nature of the Q_B protein might prevent transport across the chloroplast membrane, even if a suitable transit peptide were provided.

A more exciting prospect comes from the observation that *Agrobacterium*-based vectors are capable of transferring and integrating DNA into the chloroplast as well as the nuclear genome⁵. If such genes were linked to chloroplast-specific promoters and possibly regions of homology with chloroplast DNA, it might prove possible to direct genes into specific places in the chloroplast genome. It might even be possible, by designing a suitable vector, to replace the genes that encode normal Q_B proteins by cloned genes for herbicide-resistant Q_B proteins.

A more down to earth problem is that gene transfer systems based on agrobacteria are available for a narrow range of dicotyledonous plants. As yet no transformation system exists for major monocot crop plants such as the cereals (but see ref. 6). So the challenge for the future is to find ways of genetically engineering these crops or to persuade the public and animals to forego the delights of cereals and eat tobacco and petunia instead. □

1. Comai, L. *et al.* *Nature* **317**, 741 (1985).

2. Van den Broek, G. *et al.* *Nature* **313**, 358 (1985).

3. Schreier, P.H. *et al.* *EMBO J.* **4**, 25 (1985).

4. Golden, S.S. & Haselkorn, R. *Science* **229**, 1104 (1985).

5. de Block, M. *et al.* *EMBO J.* **4**, 1367 (1985).

6. Jones, M.G.K. *Nature New and Views* **317**, 579 (1985).

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Palaeontology

Relationships between reptiles

from T.S. Kemp

PALAEONTOLOGY has come under increasing criticism because of the alleged lack of objectivity and rigour. The emergence of cladistic analysis — a taxonomic theory that aims to provide an objective basis for recognizing relationships between organisms — has been an important development but one that is not yet accepted by all palaeontologists. Moreover, evolutionary scenarios about how and why particular transitions occurred are frequently dismissed as no more than speculative story-telling of no scientific value. It is interesting to look at two important recent papers on fossil reptiles (Carroll, R.L. and Gaskill, P. *Phil. Trans. R. Soc. B* **309**, 343; Chatterjee, S. *ibid.* 395) in the light of such attacks. Both are written in the traditional mould of palaeontology, with detailed anatomical description, discussion of evolutionary relationships that include recognition of ancestry but not formal cladistic analysis, and interpretation of functional anatomy and evolution.

Carroll and Gaskill have described a species of a marine reptile from the Middle Triassic of Italy, a member of the Nothosauria. Nothosaurs are generally smaller, much more primitive and earlier than the more familiar marine reptile group of the Mesozoic, the plesiosaurs, and for many years it has been assumed that nothosaurs are the ancestors or at least the closest known relatives of the plesiosaurs. One of the authors' main purposes in this paper is to use the structure of nothosaurs to elucidate the origin of the highly specialized locomotory mechanism of plesiosaurs.

The nothosaurs have a long trunk, limbs that are reduced in size but are still basically recognizable as tetrapod and a powerfully built tail. By analogy with modern aquatic reptiles such as crocodiles and certain iguanid lizards, the authors suggest that the basic swimming action consisted of lateral undulations of the tail and trunk, and the limbs were only for steering. The shoulder girdle of nothosaurs is surprisingly robust, however, so they propose that the animal could also move on land, by dragging its body forwards using the forelimbs — analogous to the terrestrial locomotion of sea lions.

Plesiosaur limbs, both fore and hind, are much larger, although they are more modified from the basic reptilian type. The trunk is shorter and apparently fairly rigid, and the tail is weak. Carroll and Gaskill's evolutionary scenario is, therefore, that the limbs and girdles of a nothosaur-like animal continued to strengthen and enlarge until they could produce locomotory thrust in water, and the need for lateral undulation of the trunk

and tail was lost.

Possible, perhaps plausible, as the hypothesis is, we must ask whether it has real scientific value. Certainly this functional analysis of locomotion in the nothosaurs, based on a combination of analogy with living reptiles and interpretation of the design features, is potentially testable: demonstration of features mechanically incompatible with the hypothesis would effectively refute it.

More dubious is the hypothesis that nothosaurs represent a stage in the evolution of the plesiosaurs. Nothosaurs and plesiosaurs possess a series of apparently derived characters in common, indicating a sister-group relationship, a conclusion reinforced in this account. It cannot be assumed that where nothosaurs differ from plesiosaurs, the nothosaur characters show the ancestral condition. This must be positively demonstrated by comparison with an unrelated outgroup, presumably of other reptiles. It emerges from



Reconstructed skeleton of the nothosaur *Pachypleurosaurus edwardsi*.

Carroll and Gaskill's account that in the postcranial skeleton, the only obviously shared derived character is the general arrangement of the shoulder girdle, and even here the similarity is not very close. Therefore, an equally plausible scenario is that the reduced size of the limbs, long trunk and powerful tail of nothosaurs are independent specializations — the common ancestor of this group and plesiosaurs would have been much less modified from normal reptiles than supposed. Indeed, a third possibility is that nothosaur locomotion evolved from a primitive version of plesiosaur locomotion. Because Carroll and Gaskill have selected one story from several equally unsupported possibilities, their choice may be accused of a lack of objectivity.

This criticism may be too restrictive to be fair. Their particular hypothesis does imply the potential existence, as fossils, of certain specifiable anatomical forms. Although a hypothesis cannot be refuted directly because of the failure to find particular kinds of fossils, the relative amount of evidential support for it may be reduced by the discovery of fossils consistent with an alternative hypothesis. For this reason scenarios of this kind can be regarded as properly scientific, although with the reservation that support for them is fairly weak. It is all too easy to demand that the level of evidence supporting a hypothesis be raised before it can be accepted as

scientifically valid. Palaeontology will always have to be satisfied with relatively weakly corroborated hypotheses of evolutionary processes, but this is better than eschewing such hypotheses altogether, as those that criticize scenarios seem to wish.

Chatterjee's contribution concerns some very well preserved specimens of an archosaurian reptile from the Late Triassic of Texas. *Postosuchus* is a large rauisuchian thecodont, some 4 metres long including the tail and standing about 2 metres high. The rauisuchians have only relatively recently been understood as a group of large carnivores that pre-dated the carnivorous dinosaurs. Indeed, much of the confusion arose from the misinterpretation of fragments of rauisuchian skeletons as those of true dinosaurs (see Benton, M. *Nature* **310**, 101; 1984). It is now clear that the group thrived during the Triassic but was replaced by the carnivorous dinosaurs at the end of that period. *Postosuchus* belongs in the family Poposauridae, which contains forms that seem to have paralleled the dinosaurs in certain respects, such as becoming facultatively bipedal.

Chatterjee's most controversial conclusion comes from his comparison between *Postosuchus* and the tyrannosaurian

dinosaurs of the Cretaceous. He finds a number of similarities which lead him to suggest a close relationship between the two groups, to the exclusion of all other dinosaurs. This implies that tyrannosaurs evolved independently of other dinosaurs and flatly contradicts the growing belief that dinosaurs are, after all, a monophyletic group within the archosaurs (Benton, M. *Nature* **312**, 599; 1984). Chatterjee lists 28 'diagnostic' characters shared between *Postosuchus* and *Tyrannosaurus*. Because he has not approached the problem cladistically, however, it is not clear which of these points of similarity are probably primitive (plesiomorphic), which can be suspected of convergence, and which are good candidates for synapomorphy and can be regarded as proper evidence for a cladistic relationship. It is therefore impossible to evaluate the quantity or quality of the evidence in support of his new hypothesis of relationships, short of re-doing the whole taxonomic exercise.

Nothing could illustrate more clearly the need for rigorous cladistic methods of analysis and expression than a paper such as this. The time has surely come when it is the application of non-cladist methodology that requires explicit justification, making cladism the normal procedure. □

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Cosmology

More missing matter mystery

from Carlos Frenk and Simon White

THE missing mass and galaxy formation were the subjects of two major conferences this summer. It may seem remarkable that a five-day symposium could be devoted to objects that have never been seen and whose mass is uncertain by 76 orders of magnitude. There is now a consensus, however, that most of the material content of the Universe is as yet unidentified and astronomers are understandably anxious to clarify its nature. It is also generally agreed that this dark matter plays a key role in forming the structures we see. The symposium*, held at Princeton, addressed the problem of the existence, abundance, location and nature of the dark matter; a related meeting†, connected with the inauguration of the new Canadian Institute for Theoretical Astrophysics (CITA), focused more specifically on galaxy formation.

Observational evidence for the presence of unseen matter in a variety of astronomical systems was extensively discussed in Princeton. J. Bahcall (Institute for Advanced Study, Princeton) summarized his dynamical measurements of the local mass density and concluded that only half the mass in the Solar neighborhood could be accounted for by known objects. On larger scales, gas motions in spiral galaxies provide unequivocal evidence that these galaxies are surrounded by haloes of dark matter. Careful modelling presented by K. Freeman (Mt Stromlo Observatory) and R. Sancisi (Gröningen) showed that the observed rotation curves in a number of disk systems are not consistent with the mass being distributed in the same way as the light; the maximum mass per unit light allowed by rotation velocities in the inner disk implies a total disk mass only 15 to 25 per cent of that required to explain velocities in the outer regions. As pointed out by V. Rubin (Carnegie Institution of Washington), mass distributions can be very similar in galaxies of quite different morphological appearance. Dark haloes around elliptical and dwarf galaxies are harder to detect because of the lack of suitable dynamical tracers. According to C. Canizares (Massachusetts Institute of Technology) the X-ray data on hot gas surrounding ellipticals are not yet conclusive except in a few special cases like M87. J. Kormendy (Dominion Astrophysical Observatory) showed an example of a very faint spiral which appears to have a dark halo, and M. Aaronson (Steward Observatory) presented new data on the masses of dwarf spheroidals. The velocity dispersions of the faintest such systems still appear to

imply the presence of dark matter. On larger scales there has been little change in the perceived situation despite the substantial increase in available data over the last decade; as reviewed by M. Davis (Berkeley) and J. Ostriker (Princeton), there is now general agreement that the total masses of groups and clusters of galaxies are about ten times that of the galaxies they contain. In one new development, M. Davis and A. Yahil (Stony Brook) used infrared data from the IRAS satellite to estimate the gravitational force exerted on the Milky Way by surrounding galaxies; assuming that matter distributed like the IRAS galaxies is responsible for our motion relative to the microwave background, Yahil estimates that this matter contributes 60 per cent of the cosmic closure density.

After consideration of the many constraints, M. Rees (Cambridge) identified three plausible classes of dark matter candidates: planet-like bodies, massive black holes and exotic forms of matter. If all galaxies are associated with as much dark matter as the galaxies in clusters, the dark matter would contribute about 10 per cent of the density required to close the Universe. As J. Audouze (Institute d'Astrophysique de Paris) stressed, this is roughly the baryon density needed to form the observed abundance of light elements in the Big Bang. J. Bahcall argued that the local missing mass, at least, must be baryonic since it has settled into a disk. According to A. Fabian (Cambridge) large amounts of dark matter may presently be forming from the X-ray emitting gas in rich galaxy clusters; up to 500 solar masses per year seems to be condensing out of the cooling flows in these systems. Current models for the early Universe have, however, reinforced the old prejudice that the Universe is flat; this would require most of its content to be non-baryonic and to be more smoothly distributed than the galaxies. The many exotic candidates suggested by theoreticians were discussed by M. Turner (Chicago). They include massive neutrinos, axions, supersymmetric partners of known particles, quark nuggets and shadow matter. Experiments designed to detect these objects were among the most exciting ideas discussed at Princeton.

Limits on optical and infrared emission from dark haloes, reported by P. van der Kruit (Leiden), can now exclude any significant population of main-sequence stars. Planet-like objects are not excluded, but although IRAS could detect them in the Solar neighbourhood, according to F. Low (Steward Observatory), no good candidates have been found so far.

The structure of dark haloes affects the shape of the galaxies they contain and the way they act as lenses for background quasars. J. Binney (Oxford) showed that triaxial haloes might produce warps and asymmetries in disks, while J.R. Gott III and E. Turner (Princeton) noted difficulties in interpreting the observed lenses and pointed out that scintillation effects could distinguish haloes made of stellar mass objects from haloes made of elementary particles. In standard cosmological models, different elementary particle candidates for the dark matter lead to different predictions for fluctuations in the microwave background and for the morphology of the large-scale galaxy distribution. V. Lukash (Moscow) reported new limits on background anisotropies from the Soviet RELIC satellite, and claimed that these and similar data rule out the standard neutrino- and baryon-dominated cosmologies. Massive neutrinos also give rise to large structures which are unlike any observed, as shown by the N-body simulations presented by S. White (Steward Observatory). White argued instead that a flat universe dominated by cold dark matter (particles such as axions, photinos or gravitinos) would form objects with the observed properties of galaxy haloes and galaxy clusters.

The formation of galaxies and their haloes was the main topic at Toronto. Rees emphasized that, although the cold dark-matter cosmology is by no means firmly established, it provides an attractive arena for galaxy formation. The formation of haloes in a cold dark-matter universe was discussed by S. White who sketched out a picture for the origin of the Hubble sequence of galaxy morphologies: spirals form by slow accretion of a disk in an isolated halo, whereas ellipticals are produced by mergers. Disk formation in this context was shown by J. Gunn (Princeton) to reproduce the observed photometric and kinematic properties of spirals. These models avoid explicit discussion of radiative and hydrodynamical energy injection during galaxy formation; Ostriker cautioned that such processes are likely to be important and could be responsible for forming much of the observed small-scale structure. In a cold dark-matter universe, feedback of this kind might result in the preferential formation of galaxies in the densest regions. Rees stressed that such a bias would cause analyses of galaxy clustering to give the erroneous impression that the Universe is open, even if it is, in fact, flat.

The attraction of the flat cold dark-matter model was not universally accepted. In particular, P.J.E. Peebles (Princeton) felt that the weight of present evidence favours an open universe, and that the coherence of the galaxy distribution on very large scales may require a non-gaussian distribution of initial fluctuations. P. Schechter (Mount Wilson and Las Campanas Observatory) pointed out that the

*"Dark Matter in the Universe", Princeton, 24–28 June 1985.
†"Galaxy Formation", Toronto, 19–21 June 1985.

efficiency of merging in a cold dark-matter universe might make it very difficult to form long-lived clusters of independent galaxies. Furthermore, galaxy formation in such a universe is expected to have occurred very recently; according to L. Cowie (Space Telescope Science Institute), present upper limits from searches for protogalaxies make it implausible that most galaxies formed more recently than a redshift of 6. In addition, observations of galaxies at high redshift, as summarized by R. Ellis (Durham), show little evidence for strong evolution.

An archaeological approach to the study of galaxy formation was advocated by Freeman. He noted indications for a disk-like, rapidly rotating extension of the stellar bulge in our own Galaxy, and for a slowly rotating, metal-poor halo population. He suggested that the former was produced during protogalactic collapse and that the latter might be debris from accreted dwarf satellites and fragments. Evolutionary effects may, however, have blurred the fossil record in galaxies. S. Tremaine (CITA) showed how collisional relaxation, instabilities of disks and of spheroidal systems, mergers and angular momentum transport by spiral arms can all have affected the galaxies we see. Such

effects must clearly be considered when comparing observations with simplified formation models.

It is clear from these two meetings that the past few years have seen significant progress in this field and that new developments are still occurring rapidly. In his summary talk at Princeton, S. Tremaine, director of CITA, analysed the present situation in terms of Kuhn's theory of scientific revolutions. The realization that we still do not know what makes up most of the Universe has thrown astronomy off the track of normal science and into a crisis. After a period of confusion, such crises are usually resolved by the adoption of a new paradigm, often one promoted by a few scientists who are either young or new to the field. It is not clear whether astronomy is now emerging from the present crisis. Tremaine warned that, according to historical precedent, a new paradigm will not be fully accepted until the adherents of the older order finally die off. □

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Opiates and opioids

Has morphine a physiological function in the animal kingdom?

from H. W. Kosterlitz

ALMOST since time immemorial the opium poppy, *Papaver somniferum*, has been known to contain substances that are potent analgesics, of which morphine is the most important. About ten years ago, two pentapeptides with analgesic activity, Met-enkephalin and Leu-enkephalin, were isolated from the brain, and a larger peptide, β -endorphin, soon joined them. These and other related opioid peptides are generally considered to be the natural ligands for the opioid receptors in animal tissues to which morphine, a plant alkaloid, also binds. Recent observations by the groups of Spector¹ and Goldstein², however, cast some doubt on this distinction by indicating that morphine is present in low concentrations in the brain and other tissues.

The binding characteristics of morphine indicate that it interacts mainly with the μ -opioid receptor, rather than the δ - or κ -receptors. This interaction is responsible for its analgesic properties. It is important, however, to note that the responses of the μ -receptors induced by morphine occur not only in those areas of the central and peripheral nervous systems which are responsible for its analgesic effects. Thus, when morphine is used therapeutically for pain relief, it will also affect the functions

of the respiratory, cardiovascular and gastrointestinal systems. Its role in the control of the endocrine system is particularly important. The influence of morphine and morphine-like compounds on behaviour and mood are of significance in the development of opiate addiction.

Whereas morphine interacts almost only with the μ -receptor, the endogenous opioids present in the nervous system are much less selective. The selectivity index for μ -binding of morphine is 0.98 — maximum possible value being 1.00 — whereas three of the opioid peptides in animal tissues have μ -selectivity indices of 0.66 or less (see table³). In this context, metorphamide is of special interest since its affinity for the μ -receptor is about 15 times greater than that of morphine, [Met] enkephalyl-Arg-Phe or β -endorphin. It is of importance to note that Met-enkephalin and dynorphin A have low selectivity indices for binding at the μ -receptors as they bind preferentially at the δ -site and κ -site, respectively.

What is the significance of the presence of morphine in animal tissues? Whereas its concentration in the skin of the toad is 3 pmol g⁻¹ tissue, it is only 0.05 pmol g⁻¹ tissue in bovine cerebral cortex¹, which is very low compared with the concentra-

tions of other μ -ligands. For instance, the concentrations of metorphamide in the cortex of the rat and guinea pig are 0.3 pmol g⁻¹ tissue and 6.6 pmol g⁻¹ tissue, respectively⁴. In the cortex of the rat, the concentration of [Met] enkephalyl-Arg-Phe is 36 pmol g⁻¹ tissue⁵. It should be added that there are large variations in the total immune-reactive morphine in bovine hypothalamus and adrenal¹. Because of this, it has to be accepted that no conclusions can at present be drawn about a possible physiological role of morphine in the central nervous systems of animals.

For an understanding of the pharmacology of morphine it is important to appreciate that only *P. somniferum* and *P. setigerum* have the enzymes necessary to generate morphine from thebaine, which is widely distributed in many *Papaver* species. There is evidence that, in *P. somniferum*, thebaine is biotransformed to morphine with intermediates of either codeinone and codeine or of oripavine and morphinone⁶. If it can be shown that in animal tissues thebaine or these intermediates can be converted by enzyme action to morphine, it would be a most important observation for our understanding of the physiology and pharmacology of opioids.

While I was considering the importance of the observations of Sydney Spector, Avram Goldstein and their colleagues, I received a letter from Dr Konrad Bloch pointing out to me that Thomas Mann in his novel *The Magic Mountain* (*Der Zauberberg*, 1924) prophesied the possible existence of endogenous opioids⁷. Here is his remarkable prophecy:

" 'Nothing different — oh well, the stuff to-day was pure chemistry', Joachim unwillingly condescended to enlighten his cousin. It seemed there was a sort of poisoning, an auto-infection of the organisms, so Dr Krokowski said; it was caused by the disintegration of a substance, of the nature of which we were still ignorant, but which was present everywhere in the body; and the products of this disintegration operated like an intoxicant upon the nerve-centres of the spinal cord, with an

Affinity and selectivity index for μ -binding of morphine and opioid peptides with prevalence at μ -, δ - or κ -binding sites³.

	Affinity for μ -binding ($K_i = \text{nM}^{-1}$)	Selectivity for μ -binding
Morphine	0.56	0.98
[Met] enkephalyl-Arg-Arg-Val-NH ₂ (metorphamide)	8.7	0.66
[Met] enkephalyl-Arg-Phe	0.29	0.60
β -Endorphin	0.49	0.52
Met-enkephalin	0.11	0.09
Dynorphin A	1.37	0.13

Selectivity index: K_i^{-1} for $\mu/(K_i^{-1}$ for $\mu + K_i^{-1}$ for $\delta + K_i^{-1}$ for $\kappa)$

effect similar to that of certain poisons, such as morphia, or cocaine, when introduced in the usual way from outside.

'And so you get the hectic flush', said Hans Castorp. 'But that's all worth hearing. What the man doesn't know! He must have simply lapped it up. You just wait, one of these days he will discover what that substance is that exists everywhere in the body and sets free the soluble toxins that act like a narcotic on the nervous system; then he will be able to fuddle us all more than ever. Perhaps in the past they were able to do that very thing. When I listen to him, I could almost think there is some truth in the old legends about love potions and the like . . .'

Palaeoecology

Distributions of desert plants

from Peter D. Moore

STUDYING the history of vegetation and climate in arid parts of the world presents far more problems than it does in damper climes. The availability of lake sediments and peat deposits in most temperate and many tropical areas has led to extensive studies of biological, geological and chemical materials that have permitted some detailed reconstructions of past environments. But although fossil lakes have been studied in some deserts, for example, the Rajasthan¹ and the Sahara², their scarcity has forced palaeoecologists to seek other sources of evidence concerning the former distribution of vegetation in these dry areas. Such sources include mammalian rubbish heaps³, or middens, and, more recently, the analysis of carbon isotope ratios either in plant and animal macrofossils or in the organic varnishes coating the desert rocks⁴.

The habit of animals such as packrats (*Neotoma* spp.) and porcupines (*Erethizon dorsatum*) to create piles of organic detritus, and the fact that these materials survive in arid climates for many thousands of years, has provided a useful opportunity for analysing past plant-distribution patterns and migration routes. Allowance must be made, however, for such factors as the dietary preferences and the foraging range of the animals concerned. In New Mexico, the course of migration of trees since the last glaciation has been followed by the analysis of the macrofossils in 13 middens found in the northern Chihuahuan Desert⁵. It is found that the pine (*Pinus edulis*) and juniper (*Juniperus scopulorum*) vegetation of the full glacial (18,000 year BP), which reflects less extreme climatic conditions, is gradually replaced by woodlands in the early Holocene, indicating mild wet winters together with warmer summers⁶. This vegetation gives way to desert grassland in the mid-Holocene and finally to present-day desert scrub. The invasion of such plants

There is little to add except that we expect many new developments in the opioid field. □

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as the creosote bush (*Larrea divaricata*) first occurred in the Sacramento Mountains just before 3,000 yr BP.

Altitudinal changes in vegetation during the Holocene have also been studied using packrat middens in the Californian Mojave Desert⁵. By studying a single hill, changes in the altitudinal boundary between creosote bush and blackbrush (*Coleogyne ramosissima*) scrub communities can be traced. Analyses of fossil middens show the arrival and expansion of *Coleogyne* at the expense of *Larrea* during the past 500 years, indicating the development of cooler, damper conditions. A degree of sophistication was added to this analysis by running control analyses of the contents of modern middens under known vegetation conditions and by applying ordination techniques to show trends in vegetation development.

Another fashionable palaeoenvironmental technique is the use of the ratio of carbon 13 to carbon 12 in fossil organic material from arid lands. The ratio of these isotopes in organic compounds derived from photosynthetic activity depends primarily on the photosynthetic mechanism used by the plant. Plants using a C₃ strategy of carbon fixation generally have isotope ratios in the region of -27 parts per thousand (p.p.t.) (relative to a standard material), whereas those using a C₄ or a crassulacean acid metabolism (CAM) strategy produce organic compounds with a ratio of around -13 p.p.t. This fact has been of some value in studies of the evolutionary history of the C₄ strategy of photosynthesis^{6,7}; it also aids in tracing changes of diet, like the switch to maize (a C₄ plant) by the American palaeo-Indians, as the isotope ratio is found in the bone collagen of herbivorous animals⁸.

The use of isotope ratios for the detection of fossil photosynthetic strategies is made more complex by the innate variability of the ratio and also by the influence of

climate. The variation induced in the carbon compounds of tree rings is less than 2 p.p.t.; this fluctuation provides another technique for climate reconstruction^{9,10}.

In arid lands, carbon ratio measurements have been conducted on plant material from fossil samples in caves and packrat middens in Nevada¹¹. In the CAM plant *Opuntia polyacantha*, for example, samples with an age of over 40,000 years had a ratio of -21.9 (indicative of C₃-type metabolism), whereas samples from 10,000 years ago gave a carbon ratio of -13.9 p.p.t. (showing the operation of the CAM system). This is taken to demonstrate the more arid conditions at the beginning of the Holocene¹¹.

This approach is limited by the availability of macroscopic fossil plant material, which is often scarce in deserts. An alternative source of organic carbon, obtained from several sites in North America and in the deserts of the Sinai Peninsula, has recently been tested⁴. The material used is the varnish that coats rock surfaces, derived from minute airborne particles of plant organic matter. This matter, used as an energy resource by micro-organisms, accumulates slowly. Dorn and DeNiro propose that the varnish has a carbon isotope ratio that reflects the balance of photosynthetic strategies in the surrounding plant community⁴, and that organic metabolites produced by microbes will continue to maintain the original ratio in a similar way to that of the bone collagen of herbivores. They put these ideas to the test by analysing modern rock varnish from a range of sites with known vegetation and climate and find that most arid sites have values between -11 and -16 p.p.t., while semi-arid and humid sites often have values below -22 p.p.t. These figures indeed reflect the balance of C₃, C₄ and CAM plants in the areas of study. Some preliminary tests on stratified material also indicate that the method is appropriate for the study of vegetational and environmental changes over many thousands of years. More detailed work is now needed on modern processes of varnish accretion and its relationship to local vegetation, but it seems that palaeoenvironmentalists now have a powerful new tool to apply to the study of desert history. □

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Marine ecology

Vent communities in Atlantic too

from Eve C. Southward

SURPRISE and great interest have been aroused by the discovery of hydrothermal vent communities, predominantly dense colonies of huge clams and red-plumed tube worms surrounding hot sulphide-rich springs in the ocean floor¹. They stand out like oases on the otherwise sparsely populated sea bed, their members exploit chemosynthesis as a source of food and include many species new to science. More recently 'vent-type' communities have also been found at cold seeps^{2,4}. Hot-vent communities have been reported only from the eastern Pacific, but the cold-vent type are more widely spread throughout the Pacific. Kenicutt *et al.*⁵ in a recent issue of *Nature* and Paull *et al.*⁶ on page 709 of this issue now report the first cold-vent communities to be found in the Atlantic.

Photosynthesis by phytoplankton is the origin of the organic compounds that are the basis of the food chain for marine life but, at 2,000 m depth, fall-out from surface production is small and there is no light to support photosynthesis. At such depths, hydrothermal vents expel hot, sulphide-rich water into cold oxygen-containing ocean bottom water; the resultant mix is ideal for sulphur-oxidizing chemoautotrophic bacteria, which can grow both in suspension in the water and on surfaces around the vents¹. Some also live symbiotically in the tissues of the largest animals in the vent communities and are important in providing them with nutrition, just as the unicellular plants that live in tissues of giant clams and other animals on coral reefs supply their hosts with products of photosynthesis.

Vent fluids contain hydrogen sulphide, other reduced inorganic compounds and methane, as well as high concentrations of metals in solution, all derived during the passage of seawater through very hot rocks under enormous pressure¹. Hence the chemical energy for carbon fixation by chemoautotrophs is considered to be 'new', as it is unconnected with sea-surface production; this contrasts with normal biogenic sulphide produced by sulphate reduction in sediments, which is the result of bacterial degradation of organic matter originating from photosynthesis.

At cold seeps, fluid is seawater or brine rich in sulphide and sometimes hydrocarbons. Both hot and cold-vent communities are characterized by dense populations of two types of bivalve mollusc (families Vesicomyidae and Mytilidae) and the curious vestimentiferan tube worms (phylum Pogonophora) which have no mouth or gut yet can grow up to 2 metres long and 30 millimetres wide. Although various other animal groups occur at vents, diversity is low and these

three can be called indicators. All three contain symbiotic bacteria.

Communities associated with hot or warm vents have been found in the East Pacific Rise, Galapagos Rift region and Juan de Fuca Ridge, and also in the Guaymas Basin (Gulf of California)⁷, all in the eastern Pacific. Communities associated with cold seeps have a wider geographical spread. The first were found off California, near San Diego; they contain only vestimentiferans. Sulphide was not detected in the water. Other cool

Comparison of ^{13}C depletion indicated by $\delta^{13}\text{C}$ in the flesh of animals from the two new sites — 600m: Louisiana; 3,266m: Florida.

	600 metres	3,266 metres
Vestimentifera	-27‰	-42.4‰
Bivalve mollusc	-35.5‰	-74.3‰
(Calyptogena)		(mytilid)

seeps are close to the hot-vent region in the Guaymas Basin⁷, off Oregon³ and in the Juan Trench, Tenryu Canyon and Sagami Bay⁴, on the western margin of the Pacific.

Although hydrothermal areas are known on the Mid-Atlantic Ridge, no hot-vent communities have been found in the Atlantic Ocean. A single large vestimentiferan was trawled at a depth of 500 metres off the coast of Surinam⁸, but the two seep sites in the Gulf of Mexico described by Kennicutt *et al.*⁵ and Paull *et al.*⁶ are the first on the eastern margin of North America at which the typical vent-type community has been found.

The two Atlantic sites are at very different depths. The first, at 600 metres off Louisiana, has obviously oily sediments

and was found by trawling after the collection of oil-stained cores. Its fauna includes two vesicomyid clam species and a vestimentiferan, but apparently no vent mytilids. The second, in 3,266 m at the foot of the Florida Escarpment, was discovered by the manned submersible Alvin; mytilids, vesicomyids and vestimentiferans were all collected².

Comparing data on $^{13}\text{C}/^{12}\text{C}$ ratios from these two sites^{5,6} shows that there is much greater depletion of ^{13}C in animals from the deep site (see table). The figure for the mytilid from the deep site is exceptional for an animal, the lowest previous measurement being -45.9‰ in a symbiont-containing pogonophore not associated with a vent⁹. The $\delta^{13}\text{C}$ for an animal's tissue is normally close to that of its food, a little fractionation taking place during digestion and assimilation. Greater fractionation, resulting in depletion of ^{13}C , occurs during photosynthetic or chemosynthetic fixation of carbon from carbon dioxide, the depletion being around 20‰ and the final value for $\delta^{13}\text{C}$ depending on the original value for the carbon dioxide used. When considering possible sources of carbon for animals at vents, it is important to obtain $\delta^{13}\text{C}$ values for all the likely sources. This very useful technique is also helping in the measurement of the contribution made by chemoautotrophic symbionts to the nutrition of non-vent animals living in reducing sediments in shallow water¹⁰. □

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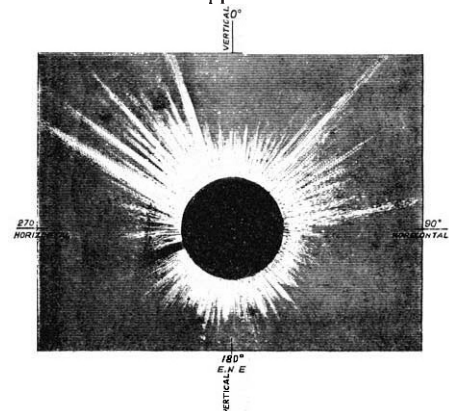
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100 years ago

THE RECENT TOTAL ECLIPSE OF THE SUN

I OBSERVED the eclipse from Tahoraite. I determined to concentrate my whole attention on the corona, and the corona alone. The moment "totality" occurred I turned my gaze towards the sun, and having previously, to save time, drawn disks on several pages of my pocket-book, I hurriedly took sketch after sketch of the shape of the corona, the rays of which were much better marked than I had been led to expect. My object in taking several sketches was to record any change in the position of the rays. I took five during the short time of totality, and their agreement is so clear as regards the number and relative position and length of the main rays, that it fully confirms the general impression left on my mind as to the fixity of this phenomenon. I was just engaged in making a last estimate of the extent of the corona between 35° and 90°, when a cry arose from the bystanders, "Look at the red flame shooting out to the left!" I withstood the temptation, and

observed the almost sudden disappearance of the corona on the reappearance of the sun.



Corona as observed during solar eclipse of September 9, 1885, as seen from Tahoraite, North Island, New Zealand, about 40 miles north of centre line of totality. (N.B. - Corona alone was observed: relative position and lengths of rays reliable: absolute lengths to be taken with caution.) from *Nature* **32** 631, 29 October 1885.

Tree death

Is lead killing German forests?

from M.H. Unsworth and R.M. Harrison

WIDESPREAD reports that trees in the forests of central Europe are damaged and dying have stimulated many hypotheses into the possible causes. Sulphur dioxide or acid rain alone do not seem to provide explanations of the distribution and symptoms of injury. Much attention at present is devoted to the possible interaction of ozone (generated photochemically in the lower atmosphere) and acidic deposition as stress factors; there are also unsubstantiated press reports that a plant virus is associated with damaged trees but not with healthy ones. Although there is speculation that damage may be caused by hitherto unrecognised trace components of the atmosphere or of rain, there have been few reports that any such substances are found in concentrations injurious to plants. On page 714 of this issue, H. Faulstich and C. Stournaras¹ now report that concentrations of trialkyl-lead in rainwater collected in the Black Forest of Germany are close to those known to kill plant and animal cells. If this is correct, it suggests that lead additives to motor fuel are directly associated with tree damage. But in our view there are strong reasons for disbelieving this interpretation.

Lead is added to gasoline (petrol) in the form of organic tetraalkyl-lead (TAL) compounds, mostly tetramethyl-lead and tetraethyl-lead, but exhaust gases contain predominantly inorganic lead aerosol. TAL vapour enters the atmosphere in small amounts through evaporation of gasoline and from exhaust pipes as unburned fuel. TAL concentrations in urban air are typically about 50 ng m^{-3} , that is, about 5% of the total lead concentration of $1 \mu\text{g m}^{-3}$. In the atmosphere, TAL is eventually converted to inorganic lead with the intermediate formation of trialkyl-lead (TriAL). If the organic compounds are not removed to the ground, the ratio of TriAL/TAL concentrations is expected to increase with distance downwind from the source. Atmospheric dispersion, however, dilutes polluted air, so that air concentrations of all lead compounds derived from gasoline are lower in rural than in urban areas.

Until recently, lack of sufficiently sensitive analytical techniques has meant that the presence of TriAL in urban and rural air has had to be inferred either from the difference between total organic lead and TAL in air^{2,3} or from any TriAL in rainwater; estimates suggest that TriAL concentrations in rural air are less than 2 ng m^{-3} . In contrast, there have been several studies of TriAL in rainwater. Since its salts are fairly soluble in water, scavenging processes in cloud and by precipitation undoubtedly provide an important sink

for removing TriAL from the atmosphere. Measurements of lead concentration in rainwater are summarized in the table. These results were obtained by three different methods: selective extraction and lead-specific detection⁴; a lead-specific gas chromatography/atomic absorption procedure^{5,7}; and a bioassay procedure for analysis of trialkyl-lead based on inhibition of tubulin polymerization in pig brain¹. The table reveals that the TriAL measurements from the Black Forest¹ are 100–1000 times larger than those from elsewhere. Are these observations correct or is the analytical method at fault?

For several reasons we find the new observations of TriAL anomalous. To

annual deposition of TriAL comparable to the total amount of lead annually added to gasoline in Germany.

Rather than seek unusual mechanisms for generating and depositing TriAL in Germany, it seems more likely that the bioassay method used by Faulstich and Stournaras is subject to interferences by other constituents of rainwater. A wide range of chemicals can inhibit the tubulin polymerization process⁸, including halothane and other anaesthetic drugs, as well as organomercurials such as methylmercury. The minor-component composition of rainwater is not well established but methylmercury and dimethylmercury, as well as many halocarbons, are certainly present in the atmosphere. The more polar compounds are no doubt also present in rainwater and it is highly likely that interferences will arise in the analytical procedure. Moreover, the method uses evaporation to concentrate rainwater samples and, if high temperatures were

Concentrations of trialkyl-lead and total lead in rainwater

Sampling location	Trimethyl-lead (ng l^{-1})	Triethyl-lead (ng l^{-1})	Total lead ($\mu\text{g l}^{-1}$)	Reference
Central Antwerp		58–330*	152–386	4
Highway (Belgium)		45–88*	148–214	4
Residential (Belgium)		40–64*	86–150	4
Rural (Belgium)		28*	29	4
University campus (Belgium)	22	10	133	5
Downtown (Belgium)	45	26	280	5
Rural (UK)	23	42	14	6
Various sites (UK)	10–31	29–71	5–34	7
Black Forest (Germany)		<2,000–62,000*	<2–210	1

*Data correspond to total trialkyl-lead

account for such high concentrations in rain would require very high atmospheric concentrations of TriAL. It is not possible to make precise calculations because chemical kinetic information is lacking, but if typical values elsewhere of $(\text{TriAL})_{\text{air}}/(\text{TriAL})_{\text{rain}}$ are $(2 \text{ ng m}^{-3})/(50 \text{ ng l}^{-1})$, then simple proportionality implies that air concentrations of $80–2500 \text{ ng m}^{-3}$ TriAL should be associated with the rain in the Black Forest. Such high concentrations of organic lead in air are found, very occasionally, but only close to gasoline filling stations² where they would be dominated by water-insoluble TAL. Mechanisms of atmospheric dispersion and rain production in clouds would not allow such concentrations in widely distributed rainfall.

The proportions of TriAL to total lead reported by Faulstich and Stournaras are unusual; the other studies listed in the table indicate that less than 1% of the total lead is in the TriAL forms. In the Black Forest, although total lead in rain is comparable to that at other non-urban sites, the proportion in the TriAL form is apparently 30–50 per cent. We cannot think of any plausible combination of exhaust emissions and deposition processes that would allow such a proportion to arise. If this occurred regularly in rain throughout Germany, it would imply an

used, many trace constituents, including TriAL, would have been broken down.

Although, in our view, the proposition that trialkyl-lead occurs in toxic concentrations in rain is entirely unproven, the fact remains that Faulstich and Stournaras appear to have observed some factor in rainwater that inhibits tubulin polymerization. If this is shown to be the case in further experiments, it should stimulate both the search to identify the mysterious factor in German rain and attempts to quantify its toxicity. □

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H. Faulstich and C. Stournaras will respond to this article within a few weeks.

An action principle for photons

SIR—I do not think it is generally known that, for a particle moving with the speed of light, the element $d\sigma = a^{1/2}dt$ is Lorentz-invariant, where a is the magnitude of the (newtonian) acceleration. Verification of this invariance by direct calculation is laborious, but comparatively simple in terms of the geometry of Minkowskian space-time. In fact the invariant pseudo arc $d\sigma$ of a null curve was found by Vessiot eighty years ago and is discussed by Bonnor² as part of the general geometry of null curves. However, these authors did not display $d\sigma$ in the very simple quasi-Newtonian form given above and I propose to derive that form here directly, so that physicists need not refer to the mathematical papers cited below^{1,2}.

Let X and Y be two 4-vectors. In terms of their components, the scalar product is

$$X \cdot Y = X_1 Y_1 + X_2 Y_2 + X_3 Y_3 - X_4 Y_4 \quad (1)$$

and this is Lorentz-invariant. Let Γ be a curve in space-time and $X(u)$ the position-vector of a general point on it, a function of a parameter u . In terms of components,

$$X = (x, y, z, ct) = (x, ct), \quad x = (x, y, z) \quad (2)$$

the 3-vector x and t being functions of u . Denoting derivatives with respect to u by subscripts, we have

$$X_u = (v, c)t_u \quad (3)$$

where v is the 3-velocity and

$$X_{uu} = (a, 0)t_u^2 + (v, c)t_{uu} \quad (4)$$

where a is the (newtonian) acceleration. The scalar product of this 4-vector by itself is

$$X_{uu} \cdot X_{uu} = A_u^2 + 2B_u t_{uu} + C_u^2 \quad (5)$$

where the coefficients are the scalar products

$$\begin{aligned} A &= (a, 0) \cdot (a, 0) = a^2 \\ B &= (a, 0) \cdot (v, c) = a \cdot v \\ C &= (v, c) \cdot (v, c) = v^2 - c^2 \end{aligned} \quad (6)$$

in accordance with (1).

So far Γ is any curve in space-time. Now taking it to be a null curve, we have $v = c$, so that $B = C = 0$ and we are left with

$$X_{uu} \cdot X_{uu} = a^2 t_u^2 \quad (7)$$

a being the magnitude of the acceleration. Hence

$$(X_{uu} \cdot X_{uu})^{1/2} du = a^{1/2} dt \quad (8)$$

Here the left hand side is Lorentz-invariant, independent of what parameter u is used. The Lorentz-invariance of the right hand side is thus established; it is the

element of pseudo-arc $d\sigma$.

Remembering that $v = c$, so that acceleration is perpendicular to velocity, we have an alternative geometrical form:

$$d\sigma = a^{1/2} dt = k^{1/2} ds \quad (9)$$

where k is the first curvature of the path in Euclidean space and ds the Euclidean element of length.

Having thus established the Lorentz-invariance of $d\sigma$, we may think of $a^{1/2}$ as a Lagrangian and consider those motions (subject always to $v = c$) which give a stationary value to the integral of $d\sigma$. Consider then two events: E_1 at position P_1 and time t_1 ; E_2 at position P_2 at time t_2 , with $t_2 > t_1$. There are three cases:

Case I. $P_1 P_2 > c(t_2 - t_1)$, so that E_2 lies outside the null cone with vertex at E_1 ; then there is no null curve connecting E_1 and E_2 .

Case II. $P_1 P_2 = c(t_2 - t_1)$, so that E_2 lies on the null cone with vertex at E_1 ; then there is a unique null curve joining E_1 to E_2 and it is straight; we have $a = 0$ and the action $A = \int d\sigma$ vanishes.

Case III. $P_1 P_2 < c(t_2 - t_1)$, so that E_2 lies inside the null cone with vertex at E_1 . This is the interesting case. There are infinitely many null curves joining E_1 to E_2 . If we think in Newtonian terms, we see that the particle has to travel a distance s which is greater than $P_1 P_2$. Thus its path must be curved, so that k is positive and the action $A = \int d\sigma$ is positive. But consider a broken path consisting of straight segments of the required total length s . This will not do, because the curvature k is undefined at a corner. Let us then employ a limiting process in which the corners are rounded off by smooth curves which disappear in the limit, giving a broken zig-zag path. If the coefficient in $d\sigma$ were k , not $k^{1/2}$, in this limit we would get a finite contribution to the action from each corner. But since the coefficient is $k^{1/2}$, the sharpened corner gives no contribution to the action.

Thus the conclusion in Case III is that the action $A (= \int d\sigma)$ attains its minimum value zero for all broken paths consisting of straight segments of total length $c(t_2 - t_1)$. In Minkowskian terms, such a broken path consists of straight null segments leading from E_1 to E_2 .

Note that in all of the above it is essential that we do not reduce space-time to two dimensions — motion on a line — for then $v = c$ implies $a = 0$. Thus the zig-zag paths considered above are not to be confused with the zig-zag paths shown by Feynman and Hibbs³.

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Nitrate and gastric cancer risks

SIR—The relationship between nitrate exposure and risk for gastric cancer is the subject of a recent paper by Forman *et al.*¹. They conclude from measurements of salivary nitrate that nitrate ingestion and gastric cancer risk are negatively correlated. Although the authors emphasize that their results reflect only the situation in Great Britain, the general message of the paper is the lack of an aetiological role for nitrate in gastric cancer. In our opinion the data do not negate such a role and the authors' inferences should not be generalized to other populations.

One of the main problems of the paper is that it leads to conclusions on the biological mechanism of gastric carcinogenesis on the basis of evidence gathered by the epidemiologic method of correlation analysis. Correlations by themselves rarely justify mechanistic inferences and are well recognized as weak instruments in the study of causality. The epidemiologic techniques have been of undeniable value in etiologic research but they face new challenges to accommodate advances in our knowledge of the mechanisms of carcinogenesis. Of special importance is the timing of genotoxic effects (which may be remote and hard to detect) and promoters (which may not be carcinogenic if acting in the wrong sequence). In the case of gastric cancer, the prevailing aetiological hypothesis calls for a complex interaction of irritants, nutritional deficiencies, mucosal atrophy, bacterial overgrowth, *in vivo* nitrosation and sequential mutations which is so intricate that it represents a special challenge to the epidemiologist.

A statement of the hypothesis linking nitrate and gastric cancer was presented in 1975² and updated in 1983³. According to this model, elevation of gastric pH above 5.5 will lead to growth of bacteria and conversion of nitrate to nitrite. Numerous studies have demonstrated that individuals at risk for gastric cancer have elevated pH and nitrite in fasting gastric juice^{4,5}. It is also well established that dietary intake of ascorbic acid can destroy gastric nitrite and prevent formation of *N*-nitroso compounds⁶. An essential component of the proposed model requires that chronic atrophic gastritis and subsequent alterations of the gastric mucosa precede nitrite formation in gastric juice³. Thus, gastritis is a *sine qua non* condition for risk of gastric cancer from nitrate. The relevant factor determining gastric nitrite is atrophic gastritis. Nitrate administered to subjects with atrophic gastritis result in nitrite levels at least 10 times greater than those found in subjects with normal mucosa⁷. It follows that a given dose of nitrate may be harmless to a normal subject but noxious to a patient with atrophic gastritis, especially if the diet contains precursors of *N*-nitroso mutagens and carcinogens^{8,9}. We seriously question the

validity of Forman, *et al.*'s estimate of 3.5 mg of nitrite derived from nitrate in high risk areas (Table 7) where the prevalence of chronic atrophic gastritis is probably considerable. The estimate ignores firm evidence of nitrate reduction in the gastric cavity in subjects with atrophic gastritis.

A second question may be raised on the validity of estimating exposure of individuals to nitrate via measurements on nitrate in saliva. Studies in Colombia¹⁰ published some years ago demonstrated a total lack of correlation between gastric cancer risk and salivary nitrate although there was a correlation with urinary nitrate. Eisenbrand *et al.*¹¹ reported exceptionally high values of nitrate and nitrite in a region in Iran which has a low rate of gastric cancer. They concluded from these data and subsequent studies in Germany that salivary flow may strongly influence salivary concentrations, such that "under cool climatic conditions . . . higher salivary flow rates will result in lower nitrite concentrations". Studies in our laboratory and others¹² have shown that salivary nitrate is not correlated with gastric nitrate, and that other ions, such as thiocyanate, may compete for the same anion transport system in salivary glands.

A third issue is the source of the nitrate. This was briefly mentioned, but underemphasized, in Forman *et al.*¹. It is well-known that vegetables are an important source of nitrate, but it makes a big difference if the vegetable carrying nitrate is a starchy root tubercle or cereal grain, which correlate positively with gastric cancer risk¹³, or green leafy vegetables and fresh fruits which correlate negatively with such risk. The carrier of the nitrate may overshadow the correlation with nitrate itself.

In balance, epidemiological studies have shown that some vegetables are protective even though they contain high quantities of nitrate. This could also be a conclusion of the article by Forman *et al.*¹. And in fact, such a conclusion would put them in total agreement with the earlier literature.

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FORMAN *ET AL.* REPLY—Tannenbaum and Correa state that the general message of our paper "is the lack of an etiologic role for nitrite in gastric cancer". What in fact we stated was "our results in general weigh against the idea that environmental nitrates play a *major* role in determining the risk of gastric cancer in Britain. They should *not*, however, be taken to imply that nitrate-related N-nitroso-compound carcinogenesis has no role in the development of gastric tumours" (our new emphasis). In other words, we clearly accept that nitrates and nitrites may have an aetiological role but not the major role in the context of Britain.

Although the distinction may appear trivial, it carries critical implications and relates directly to the role of epidemiology in our understanding of cancer. We agreed with Tannenbaum and Correa in recognizing the limitations of epidemiological methods when dealing with sophisticated multicausal models of carcinogenesis such as they and their colleagues have advanced in the area of gastric cancer. It would of course be futile to expect epidemiology to provide a complete description of the role of nitrates and nitrites in a complex pathway of events that involves numerous other interacting variables. However if nitrate exposure is a *crucial* factor in the development of gastric cancer, epidemiologists can legitimately ask the question of whether populations that experience a lot of gastric cancer have a high nitrate exposure (as we did in our paper) or alternatively whether populations exposed to a lot of nitrate experience an excess of gastric cancer (as in our recent study of fertilizer workers, manuscript in preparation). A negative answer to such questions does not mean that nitrates have no role, only that they are not a rate limiting factor and cannot explain the geographic distribution of the disease.

One has therefore to look for additional factors that are associated with an increased risk of gastric cancer. These other factors may also be dietary in origin and may be responsible for the development of atrophic gastritis or they may be the nitrosatable substrates that will react with nitrite to form N-nitroso compounds. Our results would suggest that, if nitrates are involved in the production of carcinogenic N-nitroso compounds and hence the aetiology of gastric cancer, then even

quite low levels of nitrate exposure may still be sufficient to have a detrimental effect. In this context it might be more practical to manipulate the other associated factors rather than attempt to reduce nitrate exposure from an already low level.

To put it another way, our estimate of 3.5 mg nitrite derived from nitrate in high risk populations may well be wrong, because of the higher prevalence of people with gastritis who would have an increased capacity for the formation of nitrite in the stomach. However, it would seem likely to be more beneficial to ascertain why these populations develop gastritis rather than to cut down an existing low exogenous nitrate level.

Tannenbaum and Correa make two further technical points, both of which we addressed in our original paper. The first is whether salivary nitrate and nitrite are valid measures of exposure. Table 8 in our paper shows a quite clear relationship between intake of dietary nitrate, from all sources and salivary nitrate and nitrite. If, as recent work by Tannenbaum and colleagues has shown, gastric juice nitrate does not correlate with salivary nitrate this again means that some factor other than exogenous nitrate is critical in determining the eventual gastric juice concentration.

The other technical point concerns sources of nitrate and whether certain types of dietary nitrate are more harmful than others. Certainly salivary nitrate reflects a global nitrate exposure and can not tell one anything about individual sources. From our dietary analysis meat-associated nitrate and nitrite are correlated with a high cancer risk (unlike vegetable or drinking water nitrate) but this is a very small proportion of total nitrate exposure. However, it can be a substantial proportion of nitrite intake, and because preserved meat will also contain nitrosatable amines which would reach the stomach simultaneously with the nitrite, it might be a specific hazard although this is not suggested by the available epidemiological data. Certainly more analysis needs to be carried out on individual foods and food groups.

In sum we agree with Tannenbaum and Correa that epidemiology is not equipped to deal with the full complexity of carcinogenic processes. Laboratory analyses can result in specific hypotheses about specific compounds, but epidemiology is the only way of fully testing the importance of these hypotheses in human populations and hence lead to informed public debate about prevention.

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The base and the noble

Walter Gratzer

The Periodic Table. By Primo Levi. Translated by Raymond Rosenthal. Michael Joseph/Schocken: 1985. Pp.233. £9.95, \$16.95.

THE language of chemistry, alone among the sciences, is shot through with antique imagery, compounded of astrology, folklore and the occult; it is the language of the alchemists and iatrochemists and of the atavistic quest for transmutation of base into noble. Each chapter in *The Periodic Table* bears the name of an element, serving sometimes to evoke its ancient symbolism and sometimes to recall an episode in the author's life. For Primo Levi was a journeyman chemist, a professional with a robust pride in his calling. Chief among the several fictional interludes in his book is a powerful fable about a travelling lead smelter, who explores the ancient Mediterranean in search of deposits, proud of his arcane craft and careless of the early death from plumbism that he knows will claim him, as it did his forbears.

Levi is one of a line of Jewish-Italian writers — Svevo, Moravia, Bassani, Carlo Levi — who have chronicled aspects of Italian society in the first half of our century, and in his first chapter, "Argon" — the obdurate, the noble gas that enters into no combinations with other elements — he describes his early life in a community, presumably now vanished, and new to me, that of the Piedmontese Jews. His forbears came from Provence by way of Spain in the fifteenth century, and many of the names reflect their origins. The name of the little town of Lunel, near Nîmes, was rendered into Hebrew as *yareakh*, the Moon, whence Jarach, a noted Jewish-Piedmontese surname. Levi grew up speaking a *patois* of Hebrew with Piedmontese inflexions and grammar: "Davidin, bat la cana, c'as sento nen le rukhod" his Aunt Regina would say to Uncle David, as they sat in a cafe in Saluzzo — "David, bang your stick, so that they don't hear your winds".

Levi became absorbed in chemistry as a schoolboy and went to study at Turin University. About this time Mussolini promulgated the notorious *Manifesto della Raza*, which debarred members of "non-Aryan races" from professional careers. Two distinguished Jewish-Italian scientists, both from Turin, Salvador Luria and Rita Levi-Montalcini, have written about the impact that this had on their lives. (Both came under the influence at the University of yet another Levi, Giuseppe, a respected histologist and a heroic opponent of the Fascists. Luria got away, to be washed up eventually on the other side of the Atlantic, while Levi-Montalcini went into hiding and set up a laboratory in her

kitchen, with Giuseppe Levi as her assistant, and there laid the foundation of her later work on embryology.) Primo Levi also found refuge in his work, having been offered a modest position as analyst in a mine outside Turin, where he embarked on a project to extract nickel (the demon) from the exhausted detritus of the plant. At a time when, as Levi says, "only a

He 2	Li 3	Be 4	B 5
Nc 10	Na 11	Mg 12	Al 13



	VIa	VIIa	
Cr 24	Mn 25	Fe 26	
Mo 42	Tc 43	Ru 44	
W 74	Rh 75	Pd 76	
U 92	Np 93	Pu 94	
Nd 60	Pm 61	Sm 62	

voluntarily deaf and blind man could have [had] any doubts about the fate reserved for the Jews in a German Europe", he immersed himself in his chemistry and took pleasure in the gallery of extraordinary characters around him. He achieved a Pyrrhic but intensely satisfying triumph in the laboratory and got the nickel out — an abundant pink precipitate with dimethylglyoxime. An aspiration is indeed, as Robert Louis Stevenson observed, a joy forever, a possession as solid as a landed estate.

Chemistry did more for Levi a year later, for in 1942 he joined a Partisan group — "the most disarmed partisans in the Piedmont, and probably the most unprepared" — and was forthwith captured. In Auschwitz, an experience of which he has written without rancour in another book, he was selected to work in the Buna-rubber factory, and so survived. With a friend (who did not), he kept starvation precariously at bay by selling to a clandestine cottage industry in the camp flints for cigarette lighters, chipped from rods of what he had managed to identify as iron-cerium alloy.

In the bleak years that followed the war Levi plied his trade, first in a paint factory ("Chromium"), where, by a *tour de force* of analytical chemistry and detective work in the company records, he found both the reason and the cure for the "livering" of batches of paint, that is to say their trans-

formation into a solid mass with the texture and aspect of offal. He set up with a friend a home analytical laboratory and took on contracts for disreputable entrepreneurs. Worst among these was a manufacturer of cosmetics, who made cheap lipstick with a soluble base, that ran. For a better, kissproof formula, alloxan was required and Levi set about synthesizing it from uric acid, to be extracted from chicken droppings; these he and his remarkably game bride collected from poultry farms and separated from earth, pebbles, feathers and lice. The preparation did not work and the excrement remained untransmuted and malodorous; so much for nitrogen.

The business failed resoundingly, and in "Vanadium" Levi finds himself back in a factory, superintending the manufacture of varnishes. Here a remarkable experience befalls him: some shipments of resin from IG-Farben are found to give rise to a product that refuses to dry. The correspondence that ensues focuses eventually on the use of an organo-vanadium catalyst, and is conducted from the German side by a certain Dr Müller; could it be, as certain quirks of spelling and style in the letters suggest, the same Dr Müller for whom Levi worked at Auschwitz? It was, and for Levi this seemed to bring the chance, long dreamed of, to confront, if only at a distance, one of Them — not, says Levi, for revenge but to exorcize a monstrous anomaly, "to re-establish the right proportions". Dr Müller, on receiving Levi's letter, together with a copy of his book about Auschwitz, displays feelings of confusion and regret, not amounting altogether to remorse, and strikes a discordant and painfully sentimental tone in his long reply. According to Oscar Wilde, a sentimentalist is one who desires the luxury of an emotion without wanting to pay for it. Such a one is Dr Müller. When he writes again and proposes a visit to Turin, Levi is dismayed. Dr Müller insists, but the story has a poignant ending, for, before the encounter can be consummated, Levi receives the announcement of the unexpected demise of Dr Lothar Müller in the sixtieth year of his life.

Horace Walpole said that life is a comedy to those who think and a tragedy to those who feel. Primo Levi does both, but he maintains, in the face of the turbulent events that he describes, a quizzical detachment. It is partly this quality that makes *The Periodic Table* such a transfixing book, and one that eludes categorization. It is learned, funny and elegiac, and with "Carbon" — a fancy that pursues the journey of a carbon atom from its place in a limestone lattice into the brain of the writer — it evanesces, leaving the smile of the alchemist to linger in the memory. □

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Supramolecular sort of small-talk

Marvin L. Hackert

**Supramolecular Enzyme Organization:
Quaternary Structure and Beyond.** By
Peter Friedrich. Pergamon/Akademiai Kiado,
Budapest:1985. Pp.300. £22.50, \$36.

CLASSICAL enzymology, with its stress
upon the separation and characterization
of pure enzymes, has contributed much to
our understanding of enzyme action and
regulation *in vitro*. Unfortunately, much
was lost in this process and often we failed
to establish how enzymes function *in vivo*.
This is the central theme of Peter Friedrich's
book, which foresees the dawning of a new
era in "cellular enzymology". The new
emphasis will be on *associations* between
interacting enzymes as we strive to
understand metabolic pathways with a
description that is molecular, but based on
a system that is cellular.

The book is intended for the non-
specialist and begins with a review of
terms to describe levels of enzyme structure
and the chemistry of protein-protein
interactions. A survey of enzyme associations
from quaternary structure through to
supramolecular assemblies follows, each
level being illustrated with several
examples and an account of the methods
used to investigate them. Thus, enzyme
organization is considered as a continuum
from simple interactions in an oligomer to
the ill-defined, transitory interactions
expected in a glycosome.

The supramolecular level of enzyme
organization refers to physical clusters of
two or more enzymes with different catalytic
activities. The general advantages of such
intermolecular organization are described,
with the emphasis upon how structural
organization can serve functional ends
(channelling, modulated kinetic parameters,
coordinate regulation and so on). Stable
multi-enzyme complexes are the simplest
systems where particles expressing more than
one catalytic activity can be isolated; multi-
enzyme conjugates, where different catalytic
activities reside on a single polypeptide chain,
presumably because of gene fusion, are also
considered in this class. (Tryptophan synthase
and the pyruvate dehydrogenase complexes are
given as examples of the former, while the
fatty acid synthase from yeast represents the
latter.) Post-translational, covalent modification
to control of enzymic activity is covered
briefly, but cascade systems are not mentioned.

Presented next are the well-defined
enzyme assemblies associated with subcellular
structures, integral membrane systems
such as the respiratory chain and scaffolded
enzyme arrays typified by the ribosome.
Beyond these is the emerging class of
loosely associating structures involving

"soluble" enzymes. It is to the promotion
of such systems that the book is dedicated.
Communication within and between enzyme
systems takes the form of weak intermolecular
interactions which constitute a sort of
biomacromolecular small-talk, a form of
molecular gossiping which enables control
signals to be sensed throughout a metabolic
pathway via transient encounters. Friedrich
describes the evidence for clustering of
"soluble" enzymes and the methods employed
to study such systems. The roles of macrocompartments
(organelles) and microcompartments are also
discussed, and here he defends the biological
value of transient enzyme associations in
creating microcompartments on the basis of
calculated enzyme collision frequencies and
catalytic turnover rates.

The book concludes with the author's
view of current trends in enzymology and
its prospects for the future. Computer
simulations of complex enzymes and
metabolic pathways will yield estimates of
the flux sensitivity of individual enzymes
in a pathway, while still recognizing that it
is the whole pathway and not an individual
enzyme whose regulation is important. Friedrich
advocates a holistic strategy, with less
importance being accorded to "unusual"
properties of enzymes observed *in vitro*.
For example, tricarboxylic-acid-cycle enzymes
in the mitochondrial matrix experience high
protein concentrations and relatively little
free water or unbound substrate, and so it
is important to mimic the cellular milieu in
experiments which test the hypothesis that
quasi-organized metabolic pathways exist
and have important roles *in vivo*. Conditions
resembling the natural ambience of the
enzyme must be employed in conjunction
with non-invasive spectroscopic and NMR
techniques to practise "cellular enzymology".

Over a fifth of the book is taken up by
the 1,351 references, almost all dating from
1981 or previous years. This has led to some
errors, but none of them are too serious,
and indeed are perhaps inevitable in such a
wide-ranging treatise. There are relatively
few works available that present such a
broad overview of enzyme organization,
and Peter Friedrich is to be commended
for undertaking such a difficult job. His
book will be invaluable reading for all
serious enzymologists. □

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New in paperback

- *The Primary Source: Tropical Forests and Our Future* by Norman Myers. Publisher is W. W. Norton, price is \$10.50, £7.50. For review see *Nature* 311, 184 (1984).
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- *Fusion* by Joan Lisa Bromberg. Publisher is MIT Press, price is \$9.95, £9.95. For review see *Nature* 302, 768 (1983).

Cookery in cloning

Michael Neuberger

DNA Cloning: A Practical Approach, Vols 1 and 2. Edited by D.M. Glover. IRL Press:1985. Vol. 1 pp.190; Vol. 2 pp.245. Each volume pbk £14, \$25; two-volume set £25, \$45.

SOME years ago, when I was at a station bookstall and just before getting on a train, I bought my first book on cloning. It was entitled *Cloning*, had been written by a biophysicist, David Shear, and was being remaindered for 40 pence. The book was not quite what I had anticipated. It describes how a middle-aged molecular biologist, himself a member of a clone, and his ex-wife Carolyn fight for equal rights for men and androids. It is possibly not surprising that the two volumes edited by David Glover are more convincing as well as being more useful to the run-of-the-mill molecular biologist; however, I also find them better reading.

The techniques of DNA cloning lie at the root of the recent developments in nucleic acid biochemistry. The first isolation of a type II restriction endonuclease in 1970 was shortly followed by the development of plasmid and bacteriophage cloning vectors. However it remains true that molecular cloning into *Escherichia coli* vectors constitutes the hard core of recombinant DNA technology, and it is this field that is the subject of the first volume of *DNA Cloning*.

There are nine chapters, written by different authors, covering various aspects of cloning, manipulating and ex-

• Since the completion of this review, Blackwell Scientific have published *Basic Cloning Techniques: A Manual of Experimental Procedures* (price £11.80, \$27), a 200-page paperback based on the course on molecular cloning run in the School of Biological Sciences at the University of Leicester. The book (edited by R.H. Pritchard and I.B. Holland) is a compilation of the experimental procedures taught at Leicester, and the format is essentially that of a course manual; it is, I suspect, designed for teaching purposes, although research workers will also find it of value. Within the IRL *Practical Approach* series (of which *DNA Cloning* is a part) there are other volumes that deal with agarose gel electrophoresis and the techniques associated with the study of transcription and translation, so *Basic Cloning Techniques* covers a somewhat larger range of material than *DNA Cloning* but, as might be expected, does so in considerably less depth. However the fact that all of these books cost less than a tube of restriction enzyme means that most laboratories will not need to agonize over which of them to buy. Michael Neuberger

pressing DNA in gram negative bacteria. Thus, for example, Kaiser and Murray describe phage λ vectors, Hanahan gives a detailed account of DNA transformation into *E. coli* and Fritz writes about oligonucleotide-directed mutagenesis. This volume also includes two chapters on cDNA cloning, the one by Huynh *et al.* being the most detailed guide to date on cloning into phage λ gt11. The editor has ensured that a consistent style and presentation is maintained from chapter to chapter, and the extremely detailed protocols are preceded by a helpful discussion of the theoretical background to the techniques and a consideration of the critical parameters.

The second volume deals with the introduction of DNA into organisms other than *E. coli*. Here one can learn the rudiments of cloning into bacilli, streptomycetes, yeast, plants, fruit flies and mammalian cell lines (are transgenic mammals to be included in a subsequent edition?). The range of the subject is clearly vast and the coverage necessarily somewhat eclectic;

any individual researcher is unlikely to have more than a passing acquaintance with more than a couple of the members of this zoo. Nevertheless, I found those chapters in my own field to be extremely useful, and enjoyed reading the detailed procedures about cloning into organisms that I have never worked with — much like browsing through travel brochures without having to undergo the inconvenience entailed in the travelling itself.

These two volumes are a strong addition to the IRL *Practical Approach* series. They are essentially cookery books that should be found in the laboratory, as well as the library — they may well merit the occasional monitoring with a Geiger counter. Their popularity has already been clearly demonstrated by the annoying tendency of the review copies to disappear from my desk and resurface a few days later in other laboratories scattered around the building. □

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What is chaos?

Ian Percival

Chaos. Edited by Hao Bai-Lin. World Scientific/Wiley: 1985. Pp.576. Hbk £50.80, \$63.50; pbk £23.30, \$29.

Universality in Chaos. Edited by Predrag Cvitanović. Adam Hilger: 1984. Pp.514. Hbk £29.50, \$55; pbk £11.95, \$19.

SCIENCE takes ordinary words and puts them to specialist use. The "force" that produces acceleration and the "energy" that is conserved are not the same as the force and energy of non-scientific literature. The same applies to "chaos".

Chaos can refer to static structures, but it is primarily about the motion of dynamical systems. It should be possible to formulate the motion mathematically, so chaos in the scientific sense does not refer to political chaos, for example. Furthermore the study of chaotic motion is mainly concerned with systems of finite order that are not subject to any kind of noisy input. There is no need for an infinite number of degrees of freedom or for external or quantum fluctuations for chaotic motion to take place. Dynamical systems in which future states are determined from the present state by very simple functional relations or differential equations can still be chaotic. How can such simple systems show any kind of motion that can be described as chaotic?

Local exponential divergence with global confinement make chaos, the states of the system being represented by points in the phase space. The divergence produces a local stretching of the phase space, but, because of the confinement, this stretching cannot continue without a

folding or chopping. Successive folding and refolding produces such complicated behaviour that it has the appearance of random motion, hence chaos.

For Hamiltonian systems the stretching in one direction is exactly compensated by a shrinking in another direction, so that area in the phase space is conserved, whereas in dissipative systems there is no such compensation. There can be chaotic motion for both, but it differs in detail, and for Hamiltonian systems the motion is sometimes called "irregular" or "stochastic" instead. Chaotic motion may appear to be ordered or regular over long periods of time, but in other cases the apparently random behaviour appears rapidly.

The history of the subject over the past few decades has been one of convergence. Separate studies in widely different fields led to similar conclusions, but only gradually was it realized that they all had features in common — biological populations, simplified models of fluids, chemical reactions, periodically perturbed mechanical structures, the asteroid belts of the Solar System and particles confined to storage rings or toroidal plasmas have all been shown to exhibit chaotic motion. Computers have been invaluable tools for numerical experiments, and the computer-produced illustrations of the behaviour of chaotic dynamical systems can be both illuminating and beautiful. Dynamics is repaying the compliment by providing new insights into approximations for numerical computation.

Chaotic motion has given us a fresh view of the foundations of statistical mechanics, provides us with a timely warning of the dangers of using linear theory as a first approximation in the study of nonlinear systems, and shows that number theory and fractals have impor-

tant roles to play in work upon dynamical systems. Chaotic motion in systems of finite order provides some hope that an understanding of turbulence might be within reach, a motivation which underlies many of the papers in the books under review. A solution of this particular problem still escapes us, however.

Because the study of chaos has so many roots, the early sources appear to be distributed as randomly among the journals as the phase points in the phase space of a chaotic system. There are some textbooks and good review articles on various topics, but because none of them adequately covers the whole field it is not easy to get an overall view of the subject.

Does Hao Bai-Lin's anthology of some of the more original papers improve the situation? Yes, with qualifications. The papers of Kolmogorov on the preservation of order in Hamiltonian systems and of Landau and of Hopf on the problem of turbulence are classics in their fields, and are difficult for the general reader to find; their inclusion is to be welcomed.

In other respects this collection can be compared to Predrag Cvitanović's, which also contains about 40 papers in the same general area. Surprisingly, the two books have only six papers in common. We can find out in both how Lorenz was led to doubt the feasibility of very long-range weather prediction, and how he related his differential equations in three dimensions to a one-dimensional map, we can admire May's introduction to simple systems with complicated dynamics and follow through to Feigenbaum's classic exposition of renormalization and universality. We find Metropolis, Stein and Stein's use of symbol sequences to study maps on the interval, Henon's numerical study of second-order quadratic maps with attractors, and Crutchfield and Huberman's studies of the effect of external noise. Neither editor does justice to Hamiltonian systems, however, whose properties are illustrated by the behaviour of the orbits of iterated area-preserving maps. Omitted from both collections are Greene's classic paper on the standard map and Henon's on the quadratic map.

Each book has an introduction, and those who like lively informality will prefer Cvitanović's. But both are useful collections, of great value for researchers in related fields, and the earlier papers provide valuable insight into the motivation behind more recent research. Libraries and specialists would do well to get hold of the two of them; for others *Universality in Chaos* is to be preferred on grounds of value for money. If Hao Bai-Lin's book is reasonably priced in China, it should attract a large new population of scientists to the burgeoning field of chaos in dynamical systems. □

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Disappearing acts in the oceans

Peter J. Smith

Lost Islands: The Story of Islands That Have Vanished from Nautical Charts.

By Henry Stommel. *University of British Columbia Press/Academic and University Publishers, London: 1985. Pp.146. \$30.50, £30.50.*

AS RECENTLY as 1982, the offices of Lufthansa Airlines in London, Boston and New York were displaying rather handsome globes upon which was to be found Ganges Island, located about a third of the way between Japan and Hawaii. The most startling fact about Ganges, however, is that it has never existed. During the nineteenth century, six sightings of land at about 31°N, 154°E were reported; and on the grounds that such a potential danger to shipping could not be ignored, the supposed island was included on hydrographic charts despite the innumerable failures of Pacific Mail steamers to confirm its reality. The non-existent island was still on the International Hydrographic Bureau's list of possible navigational hazards as late as 1932 and was not deleted from British Admiralty charts until 1941.

This is an example of what Stommel means by a lost island, although "lost" is perhaps hardly the most appropriate word to describe something that was never really found in the first place. Be that as it may, the version of Admiralty Chart 2683 dated 1875 contained no fewer than 142 of them in the Pacific alone. Some years later, Sir Frederick Evans, newly appointed hydrographer to the Royal Navy, decided to consign 123 pin-pricks of land to oblivion, including three genuine islands that had later to be reinstated. But many more throughout the world were to cling tena-

ciously to their ghostly life; and one (Thompson Island) is still to be found on a current US Defense Mapping Agency chart (last revised 1965) in spite of having been expunged from the British Admiralty maps as long ago as 1931.

That imaginary islands should still have been entering the charts in the nineteenth century (the starting point of Stommel's survey) is not surprising. Many were genuine mistakes arising from the continued inability to determine longitude with any accuracy. A real island could easily acquire an illusory twin, or even multiply into an archipelago, as a result of incorrect longitudes assigned by successive observers. Other fictitious islands were born of reporting, charting and typographical errors. Some were based on optical illusions; a night-time rain squall or large icebergs can convince a whole ship's crew that they are seeing great black cliffs surrounded by breakers. Then there were deliberate fabrications. Captain Benjamin Morrell, "the biggest liar in the Pacific", invented Morrell Island in the 1820s to impress his backers, yet the island remained on Chart 2683 until 1907.

Stommel, a scientist at Woods Hole Oceanographic Institute, half-apologetically dismisses his interest in the origin, history and demise of lost islands as "frivolous". He should have no such reservation. He may have entered a byway of geographical exploration, but he has made an entertaining and scholarly study of it. Besides, some imaginary islands have had far from frivolous consequences. The Mexicans put so much time, effort and money into the search for two non-existent "gold" and "silver" islands in the Pacific that they left the serious development of California far too late. As a result, the region was so tenuously Mexican by 1848 that it all too easily slipped into American hands. □

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"London going out of Town — or — The March of Bricks & Mortar", by George Cruikshank (1829), reproduced from *The English Landscape: Past, Present, and Future* edited by S.R.J. Woodell. Publisher is Oxford University Press, price is £15.

Quasi-periodic oscillations in bright galactic-bulge X-ray sources

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Quasi-periodic oscillations with frequencies in the range 5–50 Hz have recently been discovered in X-rays from two bright galactic-bulge sources and ScoX-1. We propose that these sources are weakly magnetic neutron stars accreting from disks in which the plasma is clumped. The interaction of the magnetosphere with clumps in the inner disk modulates the accretion rate, causing oscillations in the X-ray flux with many of the observed properties.

QUASI-PERIODIC oscillations have recently been discovered in X-rays from the bright galactic sources GX5-1 (refs 1, 2), CygX-2 (refs 3, 4) and ScoX-1 (refs 5, 6). The frequency, f_p , of the principal peak in the power spectrum varies from 20 to 36 Hz in GX5-1, from 28 to 45 Hz in CygX-2, and from 6 to 17 Hz in ScoX-1. In all the available data on GX5-1 and CygX-2, f_p shows a strong positive correlation with the 1–18 keV count rate. The full-width at half-maximum (FWHM) Δf_p of the peak in the averaged power spectrum at f_p varies from 4 to 13 Hz in GX5-1 and from 12 to 20 Hz in CygX-2, depending on the count rate, with relative widths $\Delta f_p/f_p$ in the range 0.21–0.60. Both sources show substantial red noise. In GX5-1 the r.m.s. fractional intensity variation caused by the quasiperiodic oscillations and that caused by the red noise both remain approximately constant as the source intensity increases, except perhaps at the highest intensities observed where the variation may be less. In CygX-2, on the other hand, the fractional variation caused by the oscillations remains approximately constant but that caused by the red noise increases as the intensity increases.

In ScoX-1, a narrow peak in the power spectrum is clearly present only during low-intensity intervals between flaring episodes and during transitions to the quiescent state. At these times the centroid frequency of the peak in the average power spectrum is strongly positively correlated with the 5–15 keV count rate, as in the other two sources (van der Klis, personal communication). However, the frequency of the oscillations appears to vary erratically during transitions to extended low states. During one transition the peak in the spectrum was observed to broaden until eventually the oscillations did not stand out above noise (Middleditch and Priedhorsky, personal communication). The FWHM of the principal peak in the ScoX-1 power spectrum varies from 1 to 10 Hz. In contrast to GX5-1 and CygX-2, the red noise down to the lowest frequencies so far explored is relatively weak when the oscillations are clearly present⁶. Whether the oscillations in ScoX-1 have the same origin as those in GX5-1 and CygX-2 is not yet clear.

It has been suggested^{8,9} that GX5-1 is a rapidly spinning but weakly magnetic neutron star accreting from a disk and that the oscillation frequency is the difference between the rotation frequency of the inner disk and the rotation frequency of the star. This beat frequency is comparable with the oscillation frequencies observed in GX5-1 (refs 8, 9), CygX-2 and ScoX-1 for neutron star spin rates ~ 100 Hz, dipole magnetic fields $\sim 10^9$ G and accretion rates $\sim 10^{18}$ g s⁻¹. Such spin rates and dipole magnetic field strengths are consistent with the hypothesis that systems like these are progenitors of the millisecond rotation-powered pulsars^{8,9}. In addition, for neutron stars rotating near their equilibrium spin rates the low harmonics of this beat frequency vary with accretion rate in a manner similar to the

observed variations of the oscillation frequency with X-ray intensity⁹.

Here we propose a physical model of the quasi-periodic oscillations which show how the beat frequency postulated previously^{8,9} can become visible in X-rays. In this model, interaction of the magnetosphere with clumps in the inner disk causes the mass accretion rate and hence the X-ray intensity to vary at a harmonic of the beat frequency given by the difference between the rotation frequency of the star and the rotation frequency of the inner disk. This idea has been suggested previously as a possible explanation for some of the quasi-periodic oscillations observed in cataclysmic variables (ref. 7; D. Lamb and B. Warner, personal communication).

We show that the power spectra given by a simple version of this model are very similar to the spectra reported in GX5-1 and CygX-2. In particular, red noise at frequencies below that of the quasi-periodic oscillations is a logical consequence of the model. If all clumps in the inner disk contribute to the oscillations and if the properties of the clumps and the nature of their interaction with the magnetosphere remain unchanged, the strength of the red noise is proportional to that of the quasi-periodic oscillations, as observed in GX5-1. If not, the strength of the red noise may increase even as the strength of the oscillations decreases, as observed in ScoX-1. We present calculations which show that X-ray pulsations caused by beaming and rotation of the neutron star are strongly suppressed relative to quasi-periodic oscillations produced according to the present model, under the conditions thought to exist in bright galactic bulge sources. Several observational tests of the model are described.

Physical model

The plasma flow pattern around a weakly magnetic neutron star accreting from a keplerian disk at near the Eddington critical rate is expected to be similar to that around supermassive black holes in galactic nuclei accreting at near-critical rates^{10–12}. The inner disk is radiation-pressure dominated, unlike the inner disks surrounding canonical accretion-powered pulsars with dipole magnetic fields $\sim 10^{12}$ G. The luminosity of the inner disk is supplied primarily by release of gravitational binding energy as the disk plasma spirals in, but also partly by radiation from the neutron star. The pressure of the radiation leaving the inner disk alone implies a disk height $h \geq (M/M_E)R$, where M_E is the mass flux corresponding to the critical accretion luminosity from the central star and R is the radius of the star. As a result, the magnetosphere is surrounded by a thick ($h \sim r$) disk corona containing cooler, denser plasma (see Fig. 1). A central corona and mass loss via a wind are expected because of heating of the surface of the disk by acoustic flux, magnetic flaring and radiation from the neutron star^{13,14}. In ScoX-1, the existence of radio lobes¹⁵ is direct evidence for mass ejection from the system.

The plasma in the inner disk is expected to be spatially inhomogeneous as a result of three mechanisms that can cause

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clumping. First, vertical eruption of magnetic flux carried inward and amplified by the accretion flow creates strong spatial inhomogeneities in the density and magnetic field strength^{14,16}. Second, for near-critical accretion rates, the disk-magnetosphere boundary lies only a few stellar radii from the surface of the neutron star; under these conditions the inner disk is expected to be thermally unstable to the formation of clumps¹⁷⁻²⁰. Third, the relative motion of magnetospheric and disk plasma drives the Kelvin-Helmholtz instability to large amplitudes near the inner edge of the disk, where the disk plasma is partially confined by the pressure of the magnetospheric field (ref. 21; J.-J. Aly, P. Ghosh and F.K.L., reported in ref. 22).

The disk ends where $B_p B_\phi \delta \approx \rho v_r r \Omega_B h$ (ref. 21). Here B_p and B_ϕ are the poloidal and toroidal components of the magnetospheric field, δ is the width of the boundary layer, ρ and v_r are the density and radial velocity of the plasma in the boundary layer, and $\Omega_B = \Omega_c - \Omega_s$ is the difference between the angular velocity Ω_c of the plasma clumps and the rotation frequency Ω_s of the star (see Fig. 2). We expect $\Omega_c \approx \Omega_{K0}$, the keplerian angular velocity at the radius²³ $r_0 = 0.52 \mu^{4/7} (2GM)^{-1/7} \dot{M}^{-2/7}$ of the boundary layer. Here μ and M are the magnetic moment and mass of the star and $\dot{M}$ is the mass flux through the boundary layer. The spread in clump angular velocities within the boundary layer is then $\Delta\Omega_B \approx |\partial\Omega_K/\partial r|_0 \delta = 1.5(\delta/r_0)\Omega_{K0}$.

In the boundary layer, plasma clumps drift radially inwards as they lose angular momentum by interaction with each other, with the surrounding gas and with the stellar magnetic field. Plasma is stripped from the clumps by interaction with the magnetospheric field and plasma. Important mechanisms are likely to include stripping by surface stresses resulting from the Kelvin-Helmholtz instability²⁴ or by reconnection of magnetic flux in the clump with the stellar flux, perhaps in a manner analogous to the reported²⁵ stripping of plasma from comets as they move through regions of alternating field direction in the solar wind. Once stripped, plasma is quickly brought into corotation with the neutron star and accretes to the stellar surface, producing X rays there.

Unless the stellar field is axisymmetrical, aligned with the rotation axes of disk and star and centred in the star, the interaction of a given clump with the magnetosphere is greater at some stellar azimuths than at others. As a result, the rate at which plasma is stripped from the clump is greater at some azimuths than others (see Fig. 3). We refer to the stellar azimuth(s) at which the stripping rate is greater for a given clump as the special direction(s) for that clump. The pattern of the special directions and the steepness of the variation of the stripping rate with azimuth affect the harmonic content of the X-ray intensity waveform produced by accretion of matter from the clump.

For a pattern of special directions with k -fold symmetry ($k = 1, 2, 3, \dots$), the pattern of mass flux to the stellar surface and hence the X-ray waveform approximately repeats with frequency $\Omega_R = k\Omega_B$. Suppose, for example, that the rotation axis of the star is aligned with that of the disk but its magnetic axis is tilted with respect to its rotation axis. For a boundary layer magnetic field that is perfectly dipolar, the magnetic pressure on a clump is $\propto 1 + 3 \cos^2(\Omega_B t + \phi)$, where ϕ is the phase of the clump. Thus, if the rate at which plasma is stripped from clumps is determined entirely by the magnetic stress acting on the clumps, $k = 2$. For the same geometry, reconnection of the magnetospheric field previously entrained by the clumps with the local magnetospheric field varies with frequency $2\Omega_B$, also giving $k = 2$. However, if there is the slightest asymmetry between the two magnetic poles, $k = 1$, even though most of the power in the clump waveform may be at $2\Omega_B$. Reconnection to the local magnetospheric field of a component of the magnetic field of a clump that remains fixed in the corotating frame (due, for example, to magnetospheric interaction with the clump) varies with frequency Ω_B , giving $k = 1$.

As long as the field in the boundary layer is nearly dipolar, the power in the principal peak of the power spectrum typically dominates that at the first overtone, due to the smoothness of

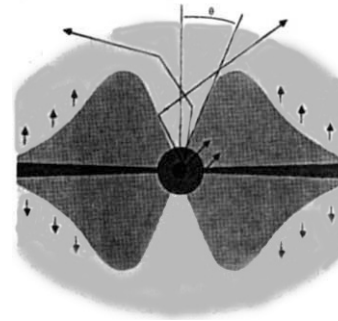


Fig. 1 Side view of the plasma flow pattern expected around a weakly magnetic neutron star accreting from a disk at near the critical rate¹². Shown are the neutron star (black circle), magnetosphere (dark shaded circle), accretion disk (dark shading), inner disk corona (moderate shading) and central corona (light shading); θ is the opening angle of the funnel of the inner disk corona. Photons (long arrows) enter the inner disk corona both from the disk and from the neutron star; photons from the neutron star emerging along the axis of the disk are scattered by plasma in the central corona. Mass is lost from the disk via a wind (short arrows).

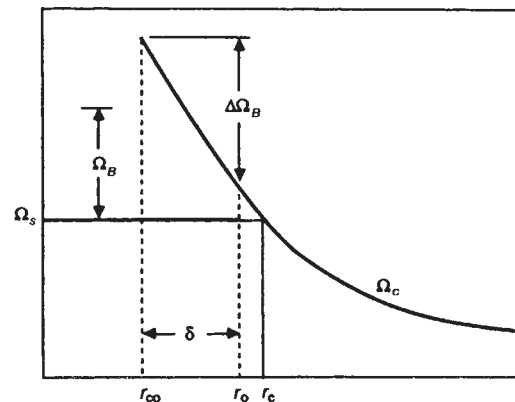


Fig. 2 Magnetospheric (Ω_s) and clump (Ω_c) angular velocities in the inner disk and boundary layer, showing the meaning of the quantities Ω_B , $\Delta\Omega_B$, r_0 and δ . Inside r_{co} , plasma is in generalized corotation⁵⁰ with the star; also indicated is the centrifugal radius r_c at which plasma in the disk corotates with the star. The width of the boundary layer has been greatly exaggerated for clarity.

the dipole field pattern. Even if the stripping rate varies as steeply as $\cos^6[(\Omega_B t + \phi)/2]$, the power in the first overtone is only 16% of that in the fundamental. Note that modulation of the X-ray intensity at the beat frequency will be observed even if the emission from the neutron star is isotropic, as the luminosity of the star is modulated.

If the pattern of radiation from the neutron star is not axisymmetrical in the disk plane at the inner disk, scattering of radiation into the line of sight by clumps in the inner disk will create a periodic anisotropy in the radiation from the system, causing some modulation of the X-ray intensity seen by a distant observer. However, we expect this effect to be much less important than modulation of the mass flux to the stellar surface for several reasons, including the small solid angle subtended by the clumps at the neutron star, the very weak beaming expected from these neutron stars, and the suppression of modulation caused by anisotropic radiation by the plasma surrounding the neutron star. The latter two effects are discussed in more detail below.

Power spectrum

The theory of random processes (see, for example, ref. 26) can be used to calculate the power density spectrum of the X-ray intensity time series $I(t)$ produced by the collection of clumps orbiting within the boundary layer. The X-ray intensity

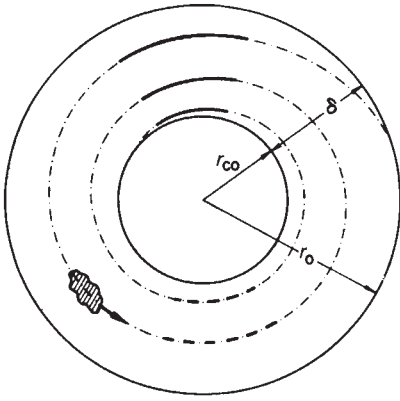


Fig. 3 Schematic drawing of the narrow boundary layer at the inner edge of the disk, from which plasma accretes onto the surface of the neutron star. The spiral depicts the trajectory of a given clump, as seen in a frame corotating with the star. In this frame, the clump moves with azimuthal velocity $r(\Omega_c(r) - \Omega_s)$ and radial velocity v_r . Plasma is stripped from the clump continuously, but the rate of stripping depends on the azimuthal position of the clump. The sections of the trajectory where the stripping rate is high for this particular clump (the 'special directions' referred to in the text) are depicted with heavy lines whereas those where it is low are depicted with light lines. In this example, the frequency of the observed oscillations is equal to the fundamental beat frequency $\Omega_B = \Omega_c(r) - \Omega_s$. If instead the pattern of special directions has 2-fold symmetry, the transfer rate is also high along those sections of the trajectory indicated with heavy dashed lines and the frequency of the observed oscillations is the first overtone of the fundamental beat frequency.

waveform produced by exactly N clumps may be written

$$I_N(t) = I_0 + \sum_{i=1}^N F(t - t_i; \phi_i) \quad (1)$$

where I_0 is a possible constant intensity, $F(t; \phi)$ is the waveform contributed by a single clump, and ϕ_i is the azimuthal phase of the i th clump at the time it enters the boundary layer. Quite generally, an arbitrary clump waveform may be expanded in harmonics of the beat frequency:

$$F(t; \phi) = \left[A + \sum_{n=1}^{\infty} B_n \cos(2\pi n f_B t + n\phi + \alpha_n) \right] G(t) \quad (2)$$

where A , B_n , ϕ , and α_n are random variables, $f_B = \Omega_B/2\pi$, and $G(t)$ is an envelope function that describes the overall shape of the waveform. Equation (2) holds even if the special directions are different for each clump, as long as they have the same symmetry and remain fixed in the frame of the star.

Assuming that the number of clumps entering the boundary layer in the observing interval is Poisson distributed, that ϕ_i is uniformly distributed, and that t_i and ϕ_i are uncorrelated, the expected power density spectrum of the time series (1) is

$$\langle P(f) \rangle = 2\langle I \rangle^2 \delta(f) + 2\langle I \rangle^2 \gamma_{RN}^2 g(f) + \langle I \rangle^2 \sum_{n=1}^{\infty} \gamma_n^2 [g(f - n f_B) + g(f + n f_B)], \quad (3)$$

where $\langle I \rangle = I_0 + \langle N \rangle \langle A \rangle$ is the mean intensity, $\gamma_{RN}^2 = \langle N \rangle \langle A^2 \rangle / 2\langle I \rangle^2$, and $\gamma_n^2 = \langle N \rangle \langle B_n^2 \rangle / 4\langle I \rangle^2$. The structure function $g(f)$ is proportional to $|s(f)|^2$, where $s(f)$ is the Fourier transform of $G(t)$, but is normalized to unit area. It includes the 'lifetime' broadening (FWHM $\Delta f_L \approx 1/\pi\tau$) produced by the finite duration τ of the waveform contributed by a single clump.

The first term in equation (3) is the power density at zero frequency due to the mean intensity, whereas the second is the power density in the Poisson noise produced by photon statistics. The term proportional to $g(f)$ describes the red noise caused by time variations in the photon arrival rate. The presence of this additional noise follows immediately from the fact that the mean X-ray intensity contributed by each clump is positive and

persists only for a finite time. All clumps that contribute to the oscillations then necessarily contribute to the red noise. (The converse need not be true.) The terms proportional to $g(f - n f_B)$ describe the power density near $n f_B$ produced by the oscillating X-ray intensity. For most waveforms, the terms proportional to $g(f + n f_B)$ can be neglected.

The repetition frequency of the oscillations in the X-ray wave train contributed by a single clump varies in time as the clump drifts inwards across the boundary layer, since its angular velocity varies with radius. This variation may be described by introducing a distribution of clump angular velocities. In addition, one expects a distribution of clump lifetimes rather than a single lifetime. These effects may be included by averaging equation (3) over the expected distributions $\rho(f_B, \tau)$, which yields equation (3) with g everywhere replaced by $\bar{g} = \langle \rho g \rangle$. In the case of the oscillation terms, $\bar{g}$ equals $\tilde{g}_n$, the convolution of g with $\rho(f_B/n, \tau)/n$. Thus, if g and ρ may both be approximated by a lorentzian (or gaussian) profile, $\tilde{g}_n$ is a lorentzian (or gaussian). After this averaging, $\langle P(f) \rangle$ has peaks at $f_n = n \tilde{f}_B$, where $\tilde{f}_B$ is the frequency $\langle \rho f_B \rangle$, with FWHMs that are, loosely speaking, the sum of Δf_L and the width $n \Delta f_S \approx n \Delta \Omega_B / 2\pi$ caused by velocity shear in the boundary layer.

The power in the zero-frequency, red noise and oscillating components is

$$P_0 = \int_0^{0+\epsilon} \langle P(f) \rangle df = \langle I \rangle^2, \quad (4)$$

$$P_{RN} = 2\langle I \rangle^2 \gamma_{RN}^2 \int_0^{\infty} \bar{g}(f) df = \langle I \rangle^2 \gamma_{RN}^2 \quad (5)$$

and

$$P_n = \langle I \rangle^2 \gamma_n^2 \int_0^{\infty} \tilde{g}_n(f) df \approx \langle I \rangle^2 \gamma_n^2 \int_{-\infty}^{\infty} \tilde{g}_n(f) df = \langle I \rangle^2 \gamma_n^2 \quad (6)$$

Thus, the r.m.s. fractional intensity variation caused by the red noise is $\gamma_{RN} = (P_{RN}/P_0)^{1/2}$ whereas that caused by the oscillation at f_n is $\gamma_n = (P_n/P_0)^{1/2}$. For comparison, the fractional modulation in the oscillation at f_n (defined as half the peak-to-peak intensity variation of a sinusoid of equivalent power, divided by $\langle I \rangle$) is $m_n = \sqrt{2} \gamma_n$.

If $F(t; \phi)$ is a pure sinusoid ($B_n \neq 0$ for only one value of n , say m) and if the stripping of each clump occurs independently, $A > B_m$ for each clump, as then the X-ray intensity contributed by each clump must be positive at all times. This implies $\gamma_{RN} > \gamma_m$. If the assumptions concerning t_i and ϕ_i made in deriving equation (3) are also satisfied, equations (5) and (6) show that P_m is at most $0.5 P_{RN}$. If $F(t; \phi)$ is a pure sinusoid but ϕ_i is not uniformly distributed, the oscillation and red noise amplitudes interfere, introducing an additional phase-dependent term $\propto \text{Re } g^*(f) g(f - m f_B)$ in equation (3) that changes the shape of the spectrum near $m f_B/2$ but alters P_m/P_{RN} by only a small amount. Such interference usually also occurs if ϕ_i is correlated with t_i . However, for some correlations between ϕ_i and t_i this interference is absent if t_i is uniformly distributed, but the oscillation Fourier amplitudes contributed by different clumps interfere constructively unlike the red noise amplitudes, causing P_m to exceed P_{RN} by a large factor. Alternatively, if $F(t; \phi)$ is a pure sinusoid but is scaled by certain functions $H(\phi)$, such as $\cos^{2q}(\phi)$ for $q \gg 1$, P_m can approach P_{RN} . Finally, if $F(t; \phi) \propto \cos^{2q}[(2\pi m f_B t + \phi)/2]$, with $q > 3$, P_m exceeds P_{RN} .

The power density $\langle P(f) \rangle$ used here is related to the power density P_f defined in ref. 27 by the expression $P_f = \langle P(f) \rangle / \langle I \rangle$. Note that although the second of equations (1) of ref. 27 displays the angular frequency ω , P_f in this equation is normalized to power per circular frequency interval.)

Suppose the principal peak in the power spectrum is at the frequency $f_p = n \tilde{f}_B$. According to the physical model described

above, as the mass flux $\dot{M}$ through the boundary layer changes, the radius r_0 of the layer changes also, causing a change in Ω_{K0} . As f_s may be treated as constant for times much shorter than the timescale for changes in the spin rate of the neutron star, here $\sim 10^5$ yr, the change in Ω_{K0} results in a change in f_p . Consider now the relation between f_p and the X-ray intensity I observed at Earth in a given energy band. If no mass was lost from the system after passing through the boundary layer, if all observed X-rays came from the surface of the neutron star and if there were no changes in the geometry of the emission region or the X-ray spectrum, I would be proportional to $\dot{M}$. However, in the bright galactic-bulge sources there are strong indications²⁸⁻³¹ that these conditions are not satisfied. We therefore consider the more general relation $I = I_E (\dot{M}/\dot{M}_E)^\beta$, where $I_E = \eta L_E$ in terms of the Eddington luminosity $L_E = 1.257 \times 10^{38} (M/M_\odot) \text{ erg s}^{-1}$ for hydrogen plasma. Here η is a conversion factor that takes into account the distance, as well as geometric and bolometric corrections. (If I is the Exosat 1–18 keV count rate, D_8 is the distance in units of 8 kpc, and if the source emits isotropically with a spectrum like GX5-1, $\eta \approx 1.42 \times 10^{-35} D_8^{-2} \text{ ct erg}^{-1}$; compare ref. 2.) Then (compare ref. 9)

$$f_p = n[a(I/I_E)^{3/7\beta} - f_s] \quad (7)$$

where $a = 1.57 \times 10^3 (B_d/10^9 \text{ G})^{-6/7} (R/10^6 \text{ cm})^{-15/7} (M/M_\odot)^{5/7}$. Here B_d is the dipole component of the stellar magnetic field. A frequently reported quantity^{2,4} is the logarithmic derivative of f_p with respect to the X-ray count-rate which, in this model, is given by (compare with ref. 9)

$$\alpha \equiv \frac{d \ln f_p}{d \ln I} \approx \frac{3}{7\beta} \frac{1}{(1 - \omega_s)} \quad (8)$$

where $\omega_s = \Omega_s/\Omega_{K0}$ is the fastness parameter²¹.

X-ray pulsations

X-ray pulsations produced by rotation of the neutron stars in the bright galactic-bulge X-ray sources have not yet been detected. The canonical view is that the magnetic fields of these neutron stars are so weak ($B_d < 10^6 \text{ G}$) that they do not channel the accretion flow (see, for example, ref. 32). In contrast, the model of quasi-periodic oscillations developed here requires neutron star magnetic fields $\sim 10^9 \text{ G}$ in the bright bulge sources exhibiting such oscillations. Such fields could channel the accretion flow to some extent³³ and hence could produce some X-ray beaming. However, in the context of the present model there are three distinct physical effects that, for distant observers, tend to suppress pulsations at the rotation frequency of the neutron star while leaving the quasiperiodic oscillations unaffected.

First, X-ray beaming from the neutron star surface is much less in the present model than in canonical accretion-powered pulsars. The relatively weak magnetic field of the neutron star is less effective in channelling the accretion flow³³. Radiation pressure forces within the magnetosphere produced by the high luminosity of the star support the accreting plasma³⁴, causing it to settle over a large fraction of the surface of the star. Evidence that accreting plasma falls over a substantial fraction of the surface comes from analyses of the hard component in the spectrum, which yield emitting areas comparable with the surface area of a neutron star^{4,30,31,35}. Such a broad distribution of accreting plasma and the resulting broad X-ray beam produces modulation that is weak and is at relatively high harmonics of the rotation frequency of the neutron star. The modulation may be further weakened by gravitational bending of the X-ray photon ray paths in the strong gravitational field of the neutron star (K. Wood, personal communication; see also ref. 52).

Second, the disk corona and central corona around the neutron star (see Fig. 1) tend to destroy X-ray pulsations caused by beaming while leaving the quasi-periodic oscillations caused by modulation of the accretion rate unaffected. The existence of a central corona with dimensions $\sim 10^7 \text{ cm}$ and optical depths ~ 4 –12 is strongly indicated by analyses of the X-ray spectra of

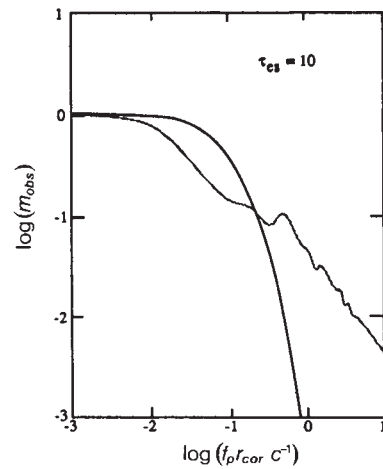


Fig. 4 Fractional modulation in quasi-periodic oscillations seen by a distant observer as a function of coronal radius r_{cor} and oscillation frequency f_p , for $\tau_{\text{es}} = 10$. Heavy curve: uniform sphere; light curve: thin shell. For the example given in the text, $\log(r_{\text{cor}} f_p c^{-1}) = -2$ (adapted from ref. 51).

the bright galactic-bulge sources^{4,31,36}. After passing through a corona of electron scattering optical depth $\tau_{\text{es}} \geq 4$, the observed fractional modulation due to beaming, m_{obs} , is much less than m_{star} , where m_{star} is the fractional modulation that would be observed in the absence of the corona (for calculations that illustrate this behaviour in a different context, see Figs 3, 5 of ref. 38). In contrast, the quasi-periodic oscillations are caused by variations in the mass flux onto the neutron star. Thus, even if the emission from the neutron star were perfectly isotropic, these oscillations would still be observable. Because they do not arise from beaming, they are not reduced by isotropization, although they can be reduced by time-of-flight smearing. However, time-of-flight smearing is unimportant if the mean time for photons to propagate through the corona is less than the oscillation period, that is, if $r_{\text{cor}} \tau_{\text{es}}/c < 1/f_p$ (see Fig. 4). Taking $r_{\text{cor}} = 10^7 \text{ cm}$, $\tau_{\text{es}} = 10$ and $m_{\text{star}} = 0.1$ as an example, the fractional modulation at the rotation frequency would be $\ll 1$ whereas the fractional modulation in 30 Hz quasi-periodic oscillations would be unaffected, that is, equal to the amplitude of the modulation in the accretion rate.

Third, inner and outer disk coronae prevent us from seeing X-rays coming directly from the neutron star except when our line-of-sight is close to the rotation axis of the star, in which case modulation caused by beaming is further weakened. The inner disk corona is expected to be both geometrically thick and optically thick to electron scattering (see the discussion of Fig. 1). There is strong evidence that in bright galactic-bulge sources the outer disk is also both geometrically and optically thick³⁸⁻⁴⁰. As a result, we do not see X-rays coming directly from the neutron star except in systems with small inclinations³². Assuming that the rotation axis of the neutron star is perpendicular to the plane of the disk, such systems are viewed from near the rotation axis, a direction in which modulation caused by beaming is absent.

Finally, the peak in the power spectrum at the rotation frequency of bright but weakly magnetic neutron star X-ray sources may appear broad (G. Hasinger, personal communication; ref. 12), unlike the peaks in the power spectra of canonical rotation-powered pulsars. If the flow of accreting plasma to the stellar surface fluctuates sharply on a timescale comparable to the spin period of the star, the central peak will be accompanied by a broad set of sidebands. Moreover, if the plasma falls on a large fraction of the stellar surface and if the brightest spots move around the surface on a timescale comparable to the spin period, the X-ray wave train will be strongly phase-modulated, broadening the peak at f_s (ref. 12).

If our analysis of the weakness of X-ray pulsations at the rotation frequency of the neutron star is correct, such pulsations

Table 1 Representative models of GX5-1

Model	n	B_d	f_s	P_s	β	$\dot{M}_1$	$\dot{M}_2$	r_{01}	r_{02}
1	1	5.5	384	2.6	3.6	0.82	0.91	3.1	3.0
2	1	19.9	88	11.4	1.0	0.50	0.70	7.4	6.7
3	1	31.6	39	26.0	0.58	0.30	0.55	11.1	9.4
4	2	12.3	191	5.2	3.6	0.82	0.91	4.9	4.7
5	2	44.6	44	22.7	1.0	0.50	0.70	11.7	10.6
6	2	70.8	19	51.8	0.58	0.30	0.55	17.6	14.9

All models assume a $1.4 M_\odot$ neutron star of radius 10^6 cm and are fit to $f_p = 20.07$ Hz and $\Delta f_p = 16.8$ Hz. The symbols and their units are: harmonic number n , dipole component of the stellar magnetic field B_d (10^9 G), stellar spin frequency f_s (Hz), rotation period P_s (ms), mass flux at 2,427 c.p.s. $\dot{M}_1$ (M_E), mass flux at 3,403 c.p.s. $\dot{M}_2$ (M_E), boundary layer radius r_{01} (10^6 cm) at $\dot{M}_1$ and r_{02} (10^6 cm) at $\dot{M}_2$. M_E is the Eddington luminosity of the neutron star.

should be most apparent in sources with relatively low intrinsic luminosities and optically thin coronae.

Discussion

The variation of oscillation frequency with X-ray intensity depends on the properties of the neutron star and the gross properties of the accretion flow, whereas the shape of the power density spectrum is determined by the physics of the boundary layer. Consider first the change in oscillation frequency.

Equation (7) fits the variation in f_p with I observed² in GX5-1 for a substantial range of neutron star magnetic fields and stellar spin rates, as illustrated by Table 1 (see also refs 2, 9). Models 1–3 assume that f_p is the fundamental beat frequency; models 4–6 that f_p is the first overtone. All assume that the photons in the so-called 'soft' component of the spectrum come from the inner disk (see Fig. 1) whereas the photons in the 'hard' component come initially from near the surface of the neutron star^{12,30,31,35} (but see ref. 4). In models 1 and 4 the 40% observed change in 1–18 keV X-ray intensity is assumed to be caused mostly by a change in the fraction of the radiation from the neutron star that is scattered into our line of sight, with the change in the mass flux through the boundary layer (and hence the accretion luminosity) only 10%. In models 2 and 5 the change in intensity is assumed to be due mostly to variation of the mass flux, which changes by 40%, whereas in models 3 and 6 the change in the mass flux (80%) is much larger than the change in the X-ray intensity, as could be the case if most of the photons from the neutron star do not reach us. For each value of β , $\dot{M}_1$ was fixed by assuming that a 1–18 keV Exosat count rate of 4,850 c.p.s. corresponds to the critical luminosity and noting that the count rate I_1 corresponding to $\dot{M}_1$ is 2,427 c.p.s. (note, however, that the value of η quoted above yields a luminosity at 4,850 c.p.s. of $1.9 L_E$ for $D = 8$ k.p.c. and $M = 1.4 M_\odot$). $\dot{M}_2$ is then determined by I_2 , which is 3,403 c.p.s. Given the range in mass accretion rate, the dipole component of the neutron star magnetic field B_d ($=2\mu/R^3$) is determined entirely by the variation Δ_p of f_p from I_1 to I_2 . Once B_d is fixed, the stellar rotation frequency f_s is determined by f_p . Both B_d and f_s are quite sensitive to the assumed variation in $\dot{M}$. The fastness parameter ω_s , which is independent of n , ranges from 0.95 for models 1 and 4 to 0.51 for models 3 and 6.

Equation (7) can also be fit to the $f_p - I$ correlation observed in CygX-2 as well as that observed in ScoX-1 during local minima of the flaring state, for similar neutron-star magnetic fields and spin rates (see also refs 4, 6). The currently allowed variation in the parameters for different models of the same source is comparable to the variation from source to source for these three systems. If the same model is used, the inferred values of B_d , r_0 , and f_s are similar for GX5-1 and CygX-2. However, B_d and r_0 are significantly smaller while f_s is significantly larger for ScoX-1 (M. van der Klis, personal communication).

Indirect support for the beat-frequency model is provided by the fact that the neutron-star rotation rates and magnetic field

strengths inferred from it are consistent with the idea^{41–43} that systems like GX5-1 and CygX-2 are progenitors of the millisecond rotation-powered pulsars^{2,8,9}. Obviously, detection of weak pulsations at the predicted neutron-star spin rate would provide the most direct evidence in favour of this model. As Table 1 shows, measurement of f_s would determine the harmonic number n and tightly constrain the change in mass flux through the boundary layer. However, it may be possible to constrain the beat-frequency model even without direct knowledge of f_s by extending the $f_p - I$ relation to higher intensity and increasing the precision of the measurements, since the shape of the theoretical $f_p - I$ curve is different for different values of the parameters.

Consider now the evidence provided by the shape of the power spectrum. In the following discussion we assume that the *average* power spectra reported for GX5-1 and CygX-2 accurately reflect the shapes of the individual spectra that have been averaged. If this is not the case, the model parameters appropriate to these sources will of course be different from those inferred here.

The width of the peak caused by quasi-periodic oscillations depends on the sum of Δf_L and $n\Delta f_s$, whereas the shape of the red noise depends only on Δf_L . Thus, fits of model spectra to observed spectra provide estimates of both quantities. Figure 5 compares one spectrum observed in GX5-1 with two model spectra. In GX5-1, the power in the oscillations is comparable to the power in the red noise down to the lowest frequencies so far explored, when the oscillations are most prominent. Recall that if the X-ray intensity waveform produced by each clump is sinusoidal and the assumptions leading to equation (3) are satisfied, P_m can be at most $0.5P_{RN}$. In the sinusoidal model P_1 does indeed equal $0.5P_{RN}$, but half the noise power is at frequencies below the lowest frequency so far explored, due to the distribution of clump lifetimes. In the other model, the waveform is not a pure sinusoid, with the result that P_1 is $1.1P_{RN}$. As shown in Fig. 5, these two models can be adjusted to fit the GX5-1 observations over the frequency range so far studied. For the non-sinusoidal model, an acceptable fit is not possible for $\Delta f_s > 0.5$ Hz. The fact that the lifetime broadening inferred from the red noise spectrum accounts for a substantial fraction of the width of the peak at f_p in the *averaged* power spectra suggests that the widths of the peaks in the *individual* spectra are not very different.

Note that if the clumps are the dominant source of accreting plasma ($I_0 \ll \langle N \rangle \langle A \rangle$) and $\langle A^2 \rangle \sim \langle A \rangle^2$, one can estimate the number of clumps in the boundary layer, as then $\langle N \rangle \sim 1/2\gamma_{RN}^2$. The result is $\langle N_a \rangle = \langle N_b \rangle \sim 30$ for the sinusoidal model and $\langle N \rangle \sim 120$ for the other. Both models can also be adjusted to give good agreement with the spectra reported⁴ in CygX-2. Because the spectra they predict have different low-frequency behaviour and different harmonic content, future observations can distinguish between them.

Analysis of the harmonic content of the oscillations can help to determine whether the observed peak is the fundamental beat frequency or an overtone. If f_p represents the fundamental ($n = 1$), power may be expected at integer multiples of f_p but not at $f_p/2$. If instead f_p is the first overtone ($n = 2$), some power may be expected at the fundamental beat frequency $f_p/2$. The distribution of power among the harmonics of f_p also constrains the physics of the process by which plasma is stripped from the clumps: the weaker the overtones, the more linear the process. The absence of a prominent feature at $2f_p$ indicates that the variation cannot be as steep as $\cos^{2q}[(2\pi f_B t + \phi)/2]$ with $q > 4$.

The ratio of the power P_p at the principal oscillation frequency to the power P_{RN} in the red noise depends on the harmonic content and fractional modulation of the X-ray waveform produced by a single clump. The relatively large apparent value of this ratio (~ 1) in GX5-1 and CygX-2 suggests a large fractional modulation. If this is confirmed when the spectrum is extended to lower frequencies, substantial azimuthal variation of the conditions in the boundary layer is indicated, favouring magnetospheric geometries in which the axis of the stellar field is

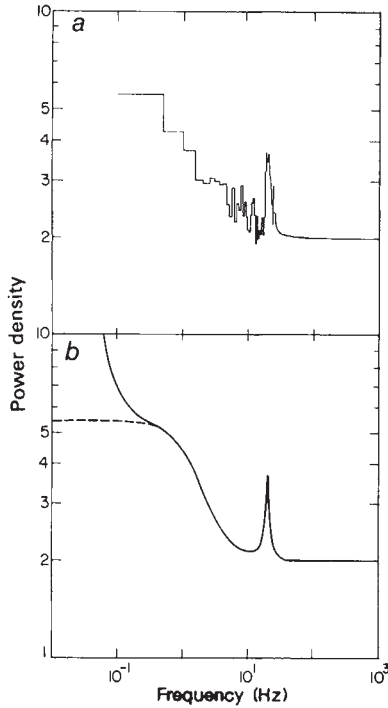


Fig. 5 *a*, Average spectrum observed in GX5-1 at 2,277–2,486 c.p.s., when $f_p = 20.07$ Hz and $\Delta f_p = 4.2$ Hz (ref. 2). The spectrum to the right of the peak has been smoothed. A visual estimate of the 1σ error at $2f_p$ for a bin size of 0.5 Hz is shown. *b*, Power spectrum calculated for a model with $F \propto \cos^6[(2\pi f_B t + \phi)/2]$ and a single clump lifetime (solid curve: $\gamma_1 = 2.5\gamma_2 = 1.5\gamma_3 = 1.1\gamma_{RN} = 6.7 \times 10^{-2}$, $f_1 = 20.07$ Hz, $\tau = 0.085$ s, $\Delta f_{S1} = 0.47$ Hz; the relative widths $\Delta \ln(f_n)$ of the peaks at f_1 , $2f_1$ and $3f_1$ are 0.21, 0.12 and 0.085, respectively) and a model with $F \propto 1 + \cos(2\pi f_B t + \phi)$ and two clump lifetimes (dashed curve: $\gamma_{RNA} = \gamma_{RNb} = \sqrt{2}\gamma_{1a} = \sqrt{2}\gamma_{2b} = 6.3 \times 10^{-2}$, $f_1 = 20.07$ Hz, $\tau_a = 50$ s, $\tau_b = 0.085$ s, $\Delta f_{Sa} = \Delta f_{Sb} = 2.6$ Hz). Both models assume the assumptions leading to equation (3) are satisfied, a clump envelope $G(t) = \theta(t) \exp(-t/\tau)$, and a lorentzian angular velocity distribution, which gives

$$g_i(f) = 2\tau_i[1 + (2\pi f\tau_i)^2]^{-1}$$

and

$$\tilde{g}_{ni}(f) = 2\tau_i(1 + \pi n \Delta f_{Si}\tau_i)[\{2\pi(f - f_n)\tau_i\}^2 + \{1 + \pi n \Delta f_{Si}\tau_i\}^2]^{-1}$$

Note the marked difference in the low-frequency behaviour of the two calculated spectra and the much smaller difference in harmonic content.

tilted by a substantial angle or is offset from the centre of the star by a substantial distance. In both sources P_p is observed to vary. If the clump waveform remains unchanged as P_p varies, P_{RN} is proportional to P_p , as observed in GX5-1 (ref. 2). More generally, P_{RN} need not be proportional to P_p and may even increase as P_p decreases, as has been reported in ScoX-1 (ref. 6). If the changes in P_p/P_{RN} are caused by variations in the shape of the clump waveform, the relative strengths of the harmonics of f_p must also change, whereas if they are due entirely to changes in the fractional modulation of the waveform, the relative strengths must remain unchanged. Thus, precise measurements of the power spectrum can show which effect is more important.

The ratios P_p/P_0 and P_{RN}/P_0 are related to the r.m.s. fractional modulation of the waveform produced by the clumps involved. The substantial (~ 4 – 10%) fractional modulations observed^{2,4,6} favour models like the present one in which the modulated X-rays come from the neutron star, as that is where the bulk of the gravitational energy becomes available. Assuming that plasma entering the inner disk accretes onto the star, the fractional modulation obtained even by converting 100% of the energy of relative motion at the magnetospheric boundary into sinusoidal X-ray oscillations would be¹² $m \leq$

$0.5(R/r_0)(1 - \omega_s)^2 \sim 0.1$ – 1% , so that dissipation of this energy is unlikely to make an important contribution to the observed oscillations. The fractional modulation obtained even by converting 100% of the energy dissipated by electrical currents in the boundary layer²¹ into sinusoidal X-ray oscillations would be¹² $m \leq (R/r_0)(1 - \omega_s) \sim 2$ – 5% , so that this effect is unlikely to be as important as modulation of the accretion rate to the stellar surface. If considerably more plasma flows through the inner disk than accretes onto the star, the energy dissipated in the boundary layer may be greater, but the variability of the X-rays from the boundary layer then tends to be smeared out by electron scattering in the plasma flowing out from the inner disk¹².

If most of the photons from the star reach us without gaining or losing appreciable energy, the oscillations and red noise should be most prominent in the spectral component that comes from the neutron star. However, photons from the star will suffer an energy shift $\Delta E \approx E$ after only $(m_e c^2/k_B T_r)$ scatterings, if $T_r > T_e$, or $(m_e c^2/k_B T_e)$ scatterings, if $T_r < T_e$ (ref. 44). Thus, if those photons from the star that reach us only after scattering through a plasma of optical depth $\tau \sim 10$ (for $T_r \approx 2$ keV and $T_e \sim 1$ – 7 keV; refs 4, 31, 36), will have energies characteristic of the temperature of the plasma rather than that of the surface of the star. As shown above, plasma of this optical depth would not affect the amplitude of the observed oscillations if its dimension is $< 10^7$ cm. In any case, the X-ray spectral dependence of the variation in intensity due to the red noise at frequencies not too far below f_p should be similar to that of the variation due to the quasi-periodic oscillations.

From the narrowness of the observed peak at f_p one can show as follows that the lifetime τ of a clump is long compared with the time for it to fall freely across the boundary layer, and hence that its angular velocity Ω_c must be keplerian. The time required for a clump to cross the boundary layer in free-fall is²¹ $\Delta t_{ff} \sim (1/\Omega_{K0}) \cdot (\delta/r_0)^{1/2}$, where Ω_{K0} is the keplerian angular velocity at r_0 . Now $\Delta f_p > \Delta f_L = 1/\pi\tau$. Further, $f_p \sim (n\Omega_{K0}/2\pi) \cdot (1 - \omega_s)$. Thus,

$$\frac{\tau}{\Delta t_{ff}} \sim \Omega_{K0} \tau \left(\frac{r_0}{\delta} \right)^{1/2} \gg \Omega_{K0} \tau > \frac{2/n}{1 - \omega_s} \frac{f_p}{\Delta f_p} \sim 10$$
– 200 (9)

Given that Ω_c is closely keplerian in the boundary layer, $\Delta f_p > n\Delta f_s = (n/2\pi) \cdot |\partial\Omega_c/\partial r|_0 \delta = (3n/4\pi)\Omega_{K0}(\delta/r_0)$. Using this relation, one can show

$$\delta/r_0 < (2/3)(1 - \omega_s)(\Delta f_p/f_p) \sim 10^{-1}$$
– 10^{-2} (10)

Noting that $v_r \sim \delta/\tau$, one can also show

$$v_r/v_\phi < (n/3)(1 - \omega_s)^2(\Delta f_p/f_p)^2 \sim 10^{-2}$$
– 10^{-4} (11)

An independent estimate of τ is provided by the noise component of the spectrum, which must flatten below the critical frequency $f_c \approx 1/2\pi\tau$.

The quasi-periodic oscillations seen in ScoX-1 are observed to disappear altogether at times (refs 5, 6). If they are correctly interpreted in terms of the present model, the fact that Δf_p has been observed to increase substantially during a transition from flaring to quiescence (W. Priedhorsky, personal communication) suggests that conditions within the boundary layer are variable. The broadening could be caused by increased shear or a shorter mean clump lifetime. Fits of equation (3) to the red noise and the oscillation peak can indicate which is more important. If conditions in the inner disk change sufficiently, the formation of clumps could be inhibited. We note also that if the density and radius of the corona above the inner disk increase to the point that $r_{cor}\tau_{cs}/c \gg 1/f_p$, the oscillations will be destroyed by electron scattering in the corona. In this case, changes in the X-ray spectrum characteristic of the development of a denser and more extensive corona should be observable. Thus, simultaneous measurements of the X-ray spectrum and power spectrum can help to determine the relative importance of these processes.

The present model suggests a natural explanation for the ‘dips’ occasionally seen in GX5-1 when the X-ray intensity is near its minimum value³⁰. In our model the radius r_0 of the

inner edge of the disk is quite close to the centrifugal radius r_c . Thus, when the mass accretion rate is near its minimum value, r_0 may briefly equal or exceed r_c . When this happens, the mass flux from the inner edge of the disk to the neutron star temporarily ceases^{21,33} and one observes only the 'soft' component from the disk³⁰.

Finally, we emphasize that the model described here may be relevant to other galactic-bulge X-ray sources. For example, the 2 Hz oscillations seen⁴⁵ in long flat-top bursts from the Rapid Burster may be a beat frequency phenomenon. If so, our model implies that the Rapid Burster has a surface magnetic field $\sim 10^{11}$ gauss, assuming $f_s \sim 20$ Hz. The absence of a so-called 'soft' component in the X-ray spectrum of the Rapid Burster (Y. Tanaka, personal communication) also indicates that this source has a significant magnetosphere. If the 10^{-3} Hz oscillations reported⁴⁶ in the 8 s accretion-powered pulsar 4U1626-67 also prove to be an example of this phenomenon⁹, it would demon-

strate that the thermally unstable region of the inner disk is not essential to the development of clumping, as such a region is absent in this source. Our model then gives an estimate of the surface magnetic field of $\sim 10^{13}$ gauss. This estimate is independent of the similar estimate derived from the observed spin-up rate⁴⁷, but consistent with it. The present work, when scaled appropriately, may also be relevant to some of the quasi-periodic oscillations observed in some cataclysmic variables^{7,48,49}.

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Hypervariable telomeric sequences from the human sex chromosomes are pseudoautosomal

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Pairing of human X and Y chromosomes during meiosis initiates within the so-called pairing region at the telomeres or the chromosome short arms. Using DNA from the Y chromosome we found sequence homology in the pairing region of the human X and Y chromosomes. This DNA is telomeric, contains repetitive sequences and is highly polymorphic in the population. The polymorphism has allowed family studies which show the sequences are not inherited as though linked to the sex chromosomes. This 'pseudoautosomal' pattern of inheritance points to an obligate recombination in the pairing region of the sex chromosomes during male meiosis.

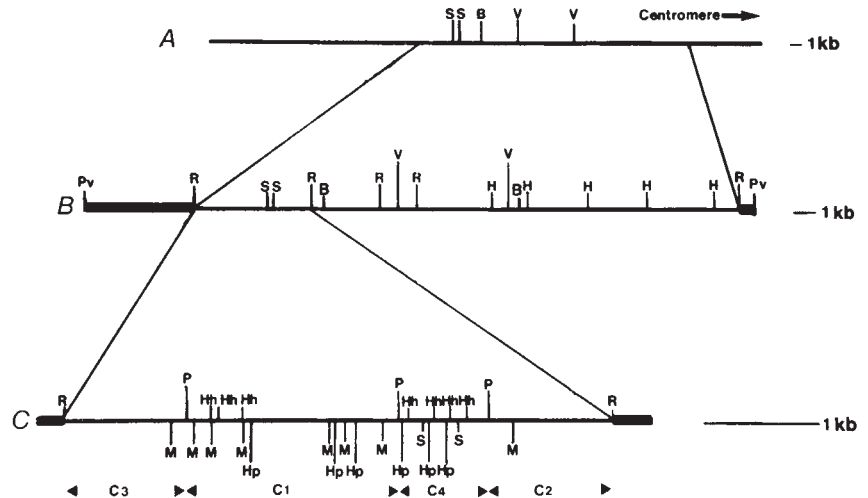
TELOMERES are DNA sequences which ensure the integrity of linear chromosomes through meiosis and mitosis. In particular, telomeric DNA is specialized to enable the 3' ends of linear chromosomal DNA to be completely replicated (see ref. 1 for review). Telomeres also have a number of cytological properties, the significance and molecular basis of which are unknown. Telomeres are often reversibly associated with one another or with the nuclear envelope. Fusion of the telomeres of different chromosomes constitutes a 'robertsonian' fusion and is a frequent cause of the karyotypic differences between related species. Understanding these features of the telomere requires

the identification and structural characterization of the DNA at its core.

Telomeric DNA has been studied in greatest detail in lower eukaryotes. *Tetrahymena* ribosomal DNA was first used as a source of telomeric DNA fragments. These telomeric sequences are G+C-rich (represented generally as 5'C₁₋₈(T/A)₁₋₄3') and appear to be sufficient for function in yeast via the addition by the yeast cell of its own C₁₋₃A version of this sequence.

In higher eukaryotes a number of telomere-associated sequences have been described¹. It is not clear how close these sequences are to the DNA terminus; most of these sequences have

Fig. 1 Restriction maps of *A*, Xp and Yg chromosome termini; *B*, cosmid CY29; *C*, expanded map of distal *EcoRI* fragment 29C. Heavy lines are vector sequences. Maps in *B* and *C* were generated by end-labelling and partial digestion. CY29 was selected from a cosmid library, constructed from a partial *MboI* digest of 3E7 DNA¹⁹ by standard techniques⁴¹ on the basis of hybridization to total human DNA. CY29 is free from detectable mouse repeated sequences, CY29 was linearized at the *PvuI* site, partially digested with the enzymes shown and transferred to nitrocellulose by bidirectional transfer⁴⁰. One filter was probed with the large *PvuI*-*EcoRI* fragment from the cosmid vector pJB8⁴² and the other with the smaller *EcoRI*-*PvuI* vector fragment. Fragment sizes were measured and calculated according to Southern and Elder⁴³. The map for 29C, a subclone in pUC9⁴⁴ was generated by cleavage with *Bam*HI, end-labelling with DNA polymerase I Klenow fragment followed by re-cleavage with *Hind*III (these sites occur only in the vector polylinker) and partial digestion with the enzymes shown. Enzymes sites are marked as follows: B, *Bam*HI; H, *Hind*III; Hh, *Hha*I; Hp, *Hpa*II; M, *Mbo*I; P, *Pst*I; Pv, *Pvu*I; R, *Eco*RI; S, *Sst*II; V, *Eco*RV.



been described on the basis of *in situ* hybridization, a technique with a maximum resolution of thousands of kilobases²⁻⁵. Many of these sequences are tandemly repeated. We describe here a human sequence present on the X and Y chromosomes, which is also telomere-associated, but which lies within 20 kilobases (kb) of the short-arm telomeres and is repeated only a few times at each telomere.

The human X and Y chromosomes, like those of many mammals, have a pairing behaviour distinct from that of the autosomes during meiosis. Pairing is initiated at Xpter and Ypter and synapsis occurs covering most of Yp and the terminal 30% of Xp⁶. Frequently a much greater extent of pairing is seen which must involve non-homologous DNA sequences⁷. Observations of XY pairing, somatic effects in XO individuals and the lack of X-inactivation of X-linked loci mapped to the pairing region of Xp (*Xg*⁸ and steroid sulphatase (*STS*)⁹) have led to the proposal that the X and Y chromosomes carry a homologous segment of DNA at Xpter and Ypter¹⁰⁻¹² and that during meiosis an obligatory recombination occurs between one chromatid of the X and Y chromosomes¹⁰. Loci distal to this recombination would be inherited as though autosomal and the term 'pseudoautosomal' has been coined to describe such a region.

Despite an intensive effort in many laboratories to provide DNA markers for the human X and Y chromosomes, no human X or Y-derived sequences which are inherited in an autosomal manner have been reported. Several sequences which are highly homologous on both sex chromosomes have been found. These sequences have arisen as a result of transposition of X-chromosome sequences onto the Y chromosome in primate evolution¹³⁻¹⁵ but to positions which are non-homologous. Furthermore, in humans the *STS* locus which maps close to the tip of Xp is clearly X-linked, implying that it must be more proximal than a site of obligatory recombination⁹. Also, *MIC2*, a human locus¹⁶ which controls expression of a cell-surface antigen 12E7, and which has alleles on Xp and Yp, shows a sex-limited expression of a polymorphism in the levels of 12E7, suggesting that *MIC2* is also proximal to any obligatory recombination site. However, support for the hypothesis comes from the finding that in the mouse the sex-reversion factor (*Sxr*), located at the tip of the Y chromosome due to rearrangement, behaves in just such a manner^{17,18}, and more recently the *STS* locus has been shown to behave pseudoautosomally on normal mouse X and Y chromosomes¹⁹.

The inheritance of human Xp and Yp telomeric sequences provides a stringent test for the existence of a pseudoautosomal region of the sex chromosomes since unless two obligate recombination events occur, these regions of the X and Y chromo-

somes would have to show an autosomal pattern of inheritance. We show here that the telomeric regions of the human X and Y chromosomes are inherited as if autosomally located, as predicted by the hypothesis¹⁰ that the X and Y chromosomes undergo an obligate crossover between one pair of chromatids during meiosis.

Cloning Y-derived sequence

We have isolated a cosmid, CY29, from a cosmid library constructed from DNA of 3E7 (ref. 20), a mouse-human hybrid with multiple Y chromosomes. This cosmid was isolated during a screen for CpG-rich sequences on the human Y chromosome. Such sequences are frequently associated with genes²¹⁻²⁴. As can be seen from the restriction map (Fig. 1c) of 29C, a subclone of CY29, sites for *Hpa*II and *Hha*I are clustered in the region of the *Sst*II sites. This would be consistent with a CpG-rich domain.

We have used a panel of somatic cell hybrid DNAs to confirm the chromosomal origin of this cosmid. A subcloned *Pst* fragment 29C1 (Fig. 1) was hybridized to a transfer of somatic cell hybrid and human DNAs as detailed in Fig. 2 legend. The hamster-human hybrid cell line 853 (Fig. 2e), which contains only a human Y chromosome by cytological criteria²⁵ and is independent in origin from the source of CY29, gives a positive signal when hybridized to 29C1. This signal is lost in a revertant of this line (Fig. 2f) lacking the Y chromosome, strongly suggesting that this sequence must be Y-derived. As well as confirming that the cosmid contains Y-chromosome sequences, these experiments showed that this sequence is also present on the X (Fig. 2b). We have extended this analysis to include two other independently derived X-only hybrids which also contain copies of this sequence (data not shown). As would be predicted, both male and female DNAs (Fig. 2c, d) also contain sequences homologous to this probe.

Cytological localization of 29C1

We have localized the sequences homologous to 29C1 using variant X and Y chromosomes in rodent-human somatic cell hybrid DNAs. Figure 3A shows the X chromosomes tested. Deletion of Xp22.32-Xpter results in loss of signal due to this probe on a Southern blot. All three intact X chromosomes and three deleted X chromosomes which retain X22.32-Xpter give a positive hybridization signal. This is consistent with at least one copy of this sequence being present at the telomere of Xp but not Xq. Localization on the Y chromosome depends on our finding that IsoY, a cell line which contains an abnormal dicentric Y chromosome fused at the short arm, gives no signal by blot analysis. This hybrid line is 12E7-positive and is positive

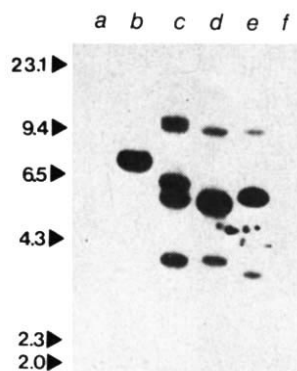


Fig. 2 X and Y localization of sequences homologous to CY29. DNA from *a*, mouse L cells; *b*, ThyB 1-33-12, a mouse-human somatic cell hybrid containing only an intact human X chromosome at the cytological level⁴⁵; *c*, XX nucleated peripheral blood cells; *d*, XY nucleated peripheral blood cells; *e*, 853, a CHO/human somatic cell hybrid containing only a human Y chromosome at the cytological level; *f*, 853 rev, a revertant of 853 selected cytologically for loss of the Y chromosome. DNA was digested with an excess of *Eco*RI and fragments separated by electrophoresis in 0.8% agarose and transferred to a nylon filter. The filter was hybridized with ~0.1 µg of 29C1 in 5×SSC, 5× Denhardt's solution, 10% dextran sulphate at 68 °C for 18 h then washed at 68 °C in 0.1×SSC, 0.05% SDS and autoradiographed with intensification. The positions of λc1857am7 *Hind*III digestion products are marked and sizes given in kb.

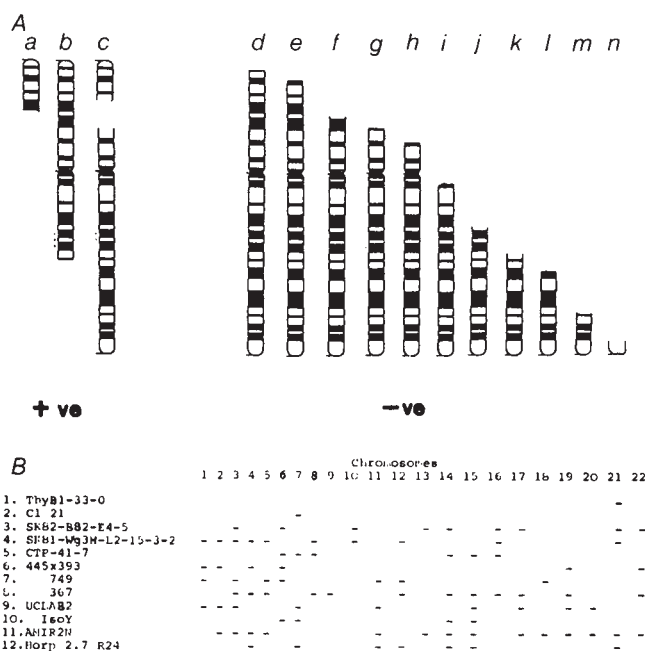


Fig. 3 **A**, Regional localization of 29C1 sequences on the X chromosomes. Hybrid cell DNAs carrying the X chromosomes illustrated were digested with an excess of *Eco*RI and analysed by electrophoresis, transfer and hybridization as described in Fig. 2 legend. The cell lines and X chromosomes tested are as follows: *a*, B2 Xpter-Xp2.1 der 21 from a female with muscular dystrophy (R. Worson, personal communication); *b*, Horl 911 R8 Xq2.2-Xpter; *c*, Sin 176 del(X)(p21.1p21.3) (ref. 46); *d*, UCLAB2 Xqter-Xp22.32 (ref. 47); *e*, 445×393 Xpter-Xp23.3; *f*, 697×175 Xqter-Xp21; *g*, 617×347 Xqter-Xp113; *h*, 422 Xqter-Xp11; *i*, 749 Xqter-Xq12; *j*, 676×175 Xqter-Xa213; *k*, 474×393 Xqter-Xq22; *l*, 791×175 Xqter-Xq23; *m*, 750 Xqter-Xw26; *n*, 367 Xqter-Xq28. **B**, Exclusion of 29C1 from autosomes. Data from Southern blot hybridizations with somatic cell hybrids in the form of a discordance table. Where a signal was detected by hybridization with 29C1 to DNA from a cell line, all chromosomes are marked +; where no signal was detected chromosomes are marked -. Cell lines were characterized by karyotype, enzyme analysis⁴⁸ and with a probe for the 12E7 locus (P. Goodfellow, personal communication).

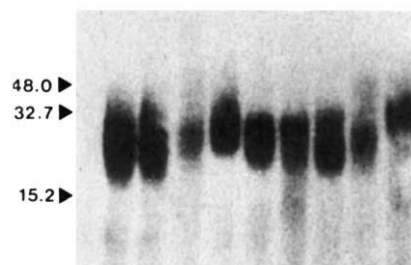


Fig. 4 Heterogeneous *Bam*HI fragments revealed by 29C1. Samples of 10 µg of *Bam*HI-digested DNA from nine samples of peripheral blood DNA were analysed on a 0.3% agarose gel by the methods described in Fig. 2 legend. Size markers were λc1857am7 *Sal*I fragments and 20 ng of this λ digest was added to each sample of *Bam*HI-digested human DNA, and after initial probing with 29C1 the filter was re-probed with λ DNA to confirm that the expected monodisperse fragments were detected (not shown).

for all Y-chromosome probes tested (P. Goodfellow and M. Rocchi, unpublished results). This is consistent with a Yp telomeric location for one or more copies of 29C1 and implies that this sequence has been deleted by a fusion event with boundaries between the telomere and at least one 12E7 locus in the IsoY cell line.

We have used a panel of somatic cell hybrid DNAs which collectively contain all autosomes but no sex chromosomes (on the basis of enzyme markers and DNA probes) to determine which, if any, autosomes carry sequences homologous to 29C1. The data in Fig. 3B show that these sequences are present only on the X and Y chromosomes.

Telomeric location of 29C1

In an attempt to find an enzyme which did not cut within this hypervariable region, we analysed *Bam*HI digests of blood DNA from several individuals. Surprisingly, this enzyme produces a smear of fragments between 18 and 23 kb in these individuals (Fig. 4). As 29C1 is unlikely to be detecting more than 10 fragments because of repetition frequency, it was apparent that this size heterogeneity was highly significant. One obvious possibility was, by analogy with trypanosome telomeres²⁶, that we are detecting the heterogeneity at a natural DNA end caused by the addition of different numbers of terminal repeats in different cells. If this were the case, such an end (1) should be sensitive to exonuclease degradation unlike non-terminal sequences and (2) at the restriction map level an apparent site for all testable restriction enzymes should be present. Finally, such a sequence should be cytologically localized to one or more of the X and Y-chromosome telomeres, a condition already supported by the data in Fig. 3.

We have used digestion with the exonuclease *Bal*31 of high relative molecular mass DNA with Southern transfer analysis to answer question (1). Figure 5A shows DNA samples from two individuals sampled after increasing times of *Bal*31 digestion, followed by *Bam*HI cleavage. A clear decrease in the size of the hybridizing fragment identified by 29C1 is evident with increasing times of digestion; ~10 kb of DNA has been removed after 2 hours of digestion. As a control this filter was hybridized simultaneously with a δ-globin DNA fragment which detects a 15.5-kb fragment in *Bam* digests of human DNA²⁷. This fragment (arrowed in Fig. 5A) shows no time-dependent decrease in size as predicted from its known position on chromosome 11²⁸. As a further control, this filter was re-hybridized with an alphoid repeat probe (Fig. 5B) showing that *Bal*31 digestion has not degraded these centromeric sequences²⁹. *Bal*31 possesses a complex range of activities besides its exonucleolytic activities³⁰. Junctions between B and Z forms of DNA helix and single-stranded regions could be responsible for the sensitivity of these sequences, although B to Z junctions would not be expected in linear deproteinized DNA in the digestion conditions used here. After prolonged times of digestion with *Bal*31,

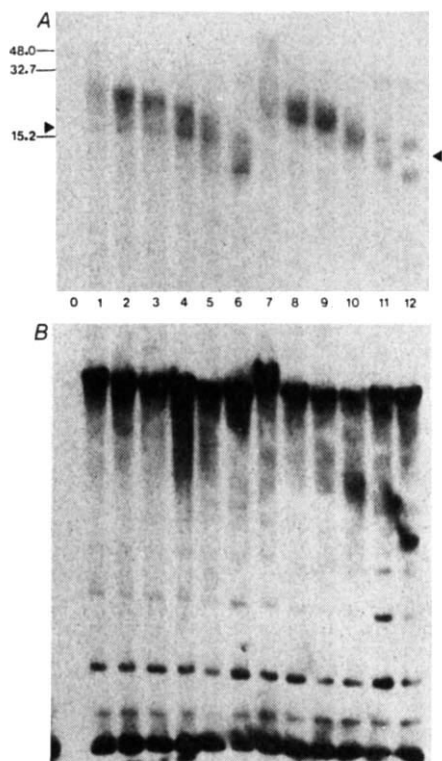


Fig. 5 *Bal31* sensitivity of 29Cl-homologous sequences. **A**, The 15.5-kb globin signal²⁷ is indicated by arrowheads and the sizes of *SalI* markers in track 0 are given in kb. The filter was reprobed with pSVX5, a plasmid containing an X chromosome-derived 2.0-kb *Bam*HI fragment composed of tandem repeats of the human alphoid sequence⁴⁵ as a further control for the *Bal31* digestion (**B**). **Methods:** Two samples of DNA from nucleated peripheral blood cells were digested with 5 µg *Bal31* at a concentration of 200 µg ml⁻¹ in 12 mM MgCl₂, 12 mM CaCl₂, 10 mM Tris, 600 mM NaCl pH 8 for 0, 4, 8, 16, 60 and 120 min (tracks 1–6 XX DNA; tracks 7–12, XY DNA). The reaction was stopped by adding 20 mM EGTA and 1 vol. water-saturated phenol. After extraction and ethanol precipitation, the DNA was digested with excess *Bam*HI (this digestion was incomplete in track 7) and the fragments fractionated on a 0.5% agarose gel. After transfer as in Fig. 2 the filter was probed simultaneously with 29Cl and a human δ -globin probe and washed as in Fig. 2.

DNAs from several individuals give hybridization patterns with 29Cl in which one or more clear bands are visible (for example Fig. 5A, lanes 6 and 12). These patterns, although reproducible for an individual DNA, differ from individual to individual, presumably reflecting the polymorphisms seen with restriction endonucleases. *Bal31* degrades G+C-rich regions more slowly than A+T-rich regions and so such a region would tend to synchronize the enzyme molecules and lead to the appearance of discrete bands.

Digests of blood DNAs with two restriction endonucleases (*EcoRV* and *SstII*) which do not cleave CY29 between the distal end of the insert and the probe but which do cleave between the probe and the proximal end of the insert, give rise to heterogeneous fragments in the same way as *Bam*HI. These fragments are also *Bal31*-sensitive. From the size of these fragments and the known positions of cleavage in CY29, we can construct the map shown in Fig. 1A. Both enzymes appear to have sites that map close to (within 0.5 kb) the *Bal31* site. This strongly supports the argument that the *Bal31*-sensitive region is a chromosome end (telomere) rather than a B/Z DNA junction. DNAs from hybrid cell lines containing human X or Y chromosomes give discrete, *Bal31*-sensitive, fragments. This implies that the telomeric sequences are homogeneous in these cells (data not shown).

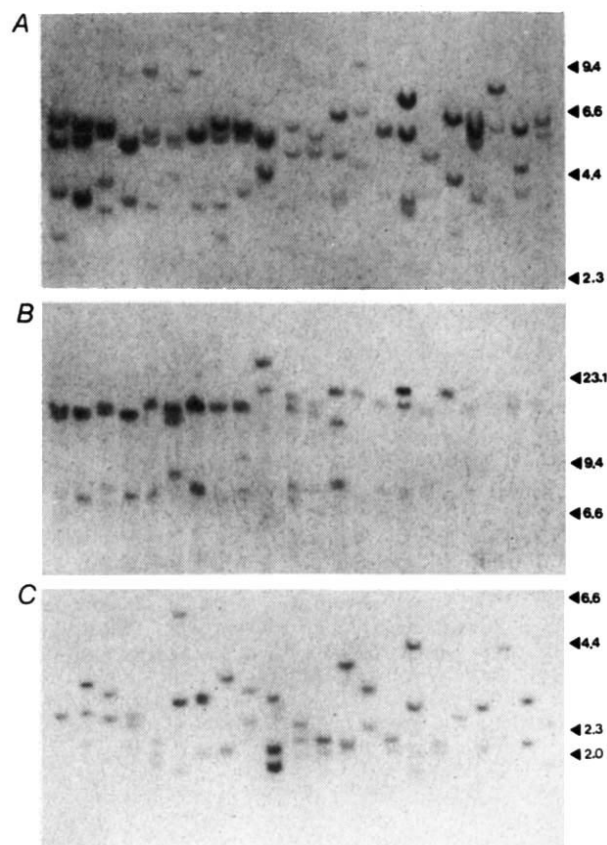


Fig. 6 Polymorphisms in human DNA: 23 DNA samples from blood of randomly selected blood donors were digested with an excess of **A**, *EcoRI*; **B**, *HindIII*; **C**, *PstI* under recommended conditions and probed with 29Cl. Transfer, hybridization, washing and size markers are as for Fig. 2.

Sequence hypervariability

It is apparent from Fig. 2 that sequences homologous to 29Cl must be present in several copies per haploid genome and that these copies must be highly polymorphic as although there are four X and two Y chromosomes represented in Fig. 2, there are no common fragments detected by probe 29Cl. We have extended this analysis in order to ask how polymorphic this sequence is and to attempt to understand the mechanism generating the polymorphisms. Figure 6 shows DNA from nucleated blood cells of 23 unrelated individuals digested with three different enzymes. It is clear that no two individuals have identical patterns. Because the polymorphisms are detected by many enzymes they cannot be due to point mutations in restriction sites; it also seems unlikely that they represent variations in the number of mini-satellite²⁵ repeats in a block because 29Cl does not hybridize in conditions of reduced stringency with two mini-satellite core sequences. Since all the *Pst* fragments (Fig. 6C) detected by this probe (which is itself a *Pst* fragment) are polymorphic this probe cannot be flanking a polymorphic region but must itself be polymorphic. We have measured the copy number of this sequence by titration and estimate that there are between 3 and 10 copies per haploid genome. There must be considerable uncertainty about this estimate because the nature of the sequence variability is unknown and could affect our measurement of copy number. It will be necessary to clone this region from a number of individuals to establish the basis of this extreme polymorphism.

Sequences 29C4 and 29C2 immediately 5' to our original probe 29Cl, detect polymorphic *HindIII* and *EcoRI* fragments as expected from the restriction map of the cosmid (Fig. 1B). These probes detect *Pst* fragments which are not polymorphic and which are identical in size in DNAs from both sexes. 29C3, in contrast, detects polymorphic fragments (data not shown).

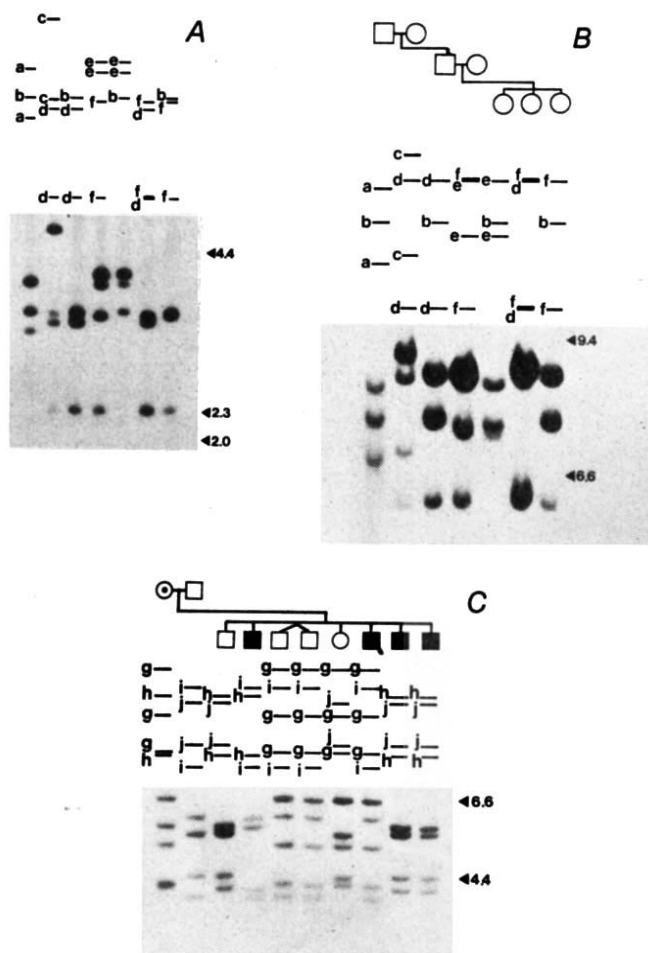


Fig. 7 Inheritance of 29Cl in two families. DNA from blood samples of individuals in the families shown were analysed as described in Fig. 2 legend. **A**, Family 1, *Pst*I; individuals are in the same order as in **B**; **B**, family 1, *Eco*RI; **C**, family 2, *Eco*RI. The results are presented schematically above the hybridization data with fragments assigned to haplotypes. Fragments detected by this sequence are sufficiently polymorphic to provide a good check for paternity. We have extended the number of individuals analysed with *Eco*RI to 83. No two individuals have identical patterns. Family 2 is affected by X-linked retinitis pigmentosa (XLRP). 29Cl shows either 1 or 3 recombinations in 4 meioses with this locus (this family is uninformative for RFLPs tightly linked to XLRP and so we are unable to determine phase).

This suggests that 29Cl defines a boundary between hypervariable and non-variable regions of the X and Y chromosomes.

Recombination between X and Y

A highly polymorphic sequence in this terminal location on the X and Y chromosomes allows a direct test of the hypothesis that an obligatory recombination event occurs between one pair of X and Y chromatids at each meiosis¹⁰. The outcome of such a process would be that alleles distal to this recombination event will be exchanged between X and Y chromosomes and will not show sex linkage in a pedigree.

We have analysed five families and found no examples of Y linkage of informative fragments in 25 meioses. In three families, however, one uninformative fragment was present in both father and mother. In Fig. 7 we present data on two fully informative families. In family 1 (Fig. **A**, **B**) we can exclude X linkage for all paternal fragments and demonstrate that two out of three daughters inherit a paternal Y chromosome fragment. The only assumption is that at least one copy of a sequence homologous to 29Cl is always present on both the X and the Y chromosomes, as suggested by our cell hybrid data. Considering only the second

two generations in the *Pst*I digest (Fig. 7A) of these DNAs, the largest fragment in the paternal sample has been inherited by daughters 1 and 3 but not by 2 and is therefore not X-linked, the next largest paternal fragment is inherited only by daughter 2 and is therefore not X-linked and the smallest paternal fragment is not inherited by the first daughter and so is also not X-linked. Thus although one at least of these fragments must be located on the X chromosome, none behave genetically as X-linked. In the *Eco*RI digest (Fig. 7B) although one fragment is uninformative in the second and third generations, one fragment can be assigned to the paternal Y chromosome. The second largest fragment (fragment b in Fig. 7B) in the grandfather is the only fragment inherited by the father and must be on the father's Y chromosome. Daughters 1 and 3 have inherited this fragment presumably as a consequence of X/Y recombination in the father. In family 2 (Fig. 7C) we cannot determine which fragments are derived from the paternal Y but no fragment is inherited by all sons. In both families there are two independently segregating sets of fragments in each individual which show a mendelian pattern of inheritance (presented schematically above the blots in Fig. 7). Fragments in both *Pst*I and *Eco*RI digests (family 1) can be fitted into the same sets which behave as haplotypes. Although the family sizes are small this supports our localization of these sequences to two loci, one on the X chromosome and one on the Y chromosome. Thus two out of three daughters carry a paternal X chromosome which is a product of X/Y recombination in the father of family 1, and three out of six sons in family 2 carry a Y chromosome which is a X/Y recombination product. This is consistent with there being an obligate recombination event. Greater numbers of informative meioses will be necessary to demonstrate this point conclusively.

Discussion

From the observations reported here and the known structures of telomeres from lower eukaryotes such as yeast and *Tetrahymena*, we can draw some conclusions about the possible nature of the telomeres of the human X and Y chromosome short arms. First, the length heterogeneity suggests that these telomeres are subject to a repeat addition process similar to that observed for trypanosome telomeres²⁶ or for *Tetrahymena* telomeres when introduced into yeast cells³². The addition process may not be a universal consequence of cell division since hybrid lines containing X and Y chromosomes show only a very limited degree of heterogeneity, if any, in the size of the terminal fragments. Addition/loss may be limited to certain cell types or stages of development.

Telomere-associated DNA has been isolated from plant⁵ and insect genomes^{2,3} as well as from lower eukaryotes. Repeated sequences seem to be generally present in these regions although the repeat frequency and complexity vary greatly. One class of such repeated sequences is the yeast Y sequence³³ which shows strong similarities in behaviour to the sequences detected by 29Cl. Almost all Y copies are sensitive to *Bal*31 digestion and thus are at telomeric locations. Different strains of *Saccharomyces cerevisiae* contain Y sequences in different copy numbers and with extensive strain to strain differences of restriction fragment length³⁴. Tetrad analysis shows that new fragments are created during meiosis, in two cases by premeiotic recombination and in one case by meiotic gene conversion. Appearance of a new fragment was accompanied by loss of a parental fragment. We suggest that at least the human Xp and Yp telomeres are also subject to such processes. No new fragments homologous to 29Cl have been observed in a study of 25 offspring in five families, suggesting that these events occur less frequently than in yeast.

The availability of chromosome components such as telomeres, centromeres and origins of replication has made the construction of synthetic yeast chromosomes possible. These chromosomes have allowed other important chromosomal features such as length and centromere position^{35,36} to be studied. Many questions about human chromosome function

could be answered in a similar way if a synthetic human chromosome could be constructed. Such a chromosome might also provide a suitable vector for the controlled addition of exogenous DNA to mammalian cells. Using probes described here it should be possible to obtain sequences almost up to the DNA terminus itself and then to start a 'building block' approach to chromosome construction.

We have shown that at least at the very tip of Xp and Yp, a region of DNA exists which is genetically pseudoautosomal¹⁰. This is consistent with the inheritance of *STS* and *Sxr* in the mouse^{12,14} and supports the hypothesis that XY pairing in meiosis is initiated due to DNA sequence homology. This contradicts Ashley's view³⁷ that this synapsis involves no sequence homology and that a recombination event does not occur. We have no evidence which allows us to estimate the size of the pseudoautosomal region. It seems probable that the region is small, since a concentrated effort to produce X and Y chromosome DNA markers in many other laboratories has only recently produced sequences which are inherited autosomally (J. Weissenbach and D. Page, personal communication). If the pseudoautosomal region is in fact small, it may be possible to determine its size by pulse field electrophoresis³⁸ and to obtain cloned DNA fragments from the region in which the obligate recombination occurs. This would provide a valuable substrate

for a molecular analysis of the mechanism of recombination in humans.

Recombination is proximal to the region detected by 29Cl in the families studied here. Recombination within this region might be expected to generate new restriction fragment lengths in recombinant progeny. It is possible that an obligate recombination is required for all pairs of homologous chromosomes in order to achieve correct segregation and that initiation of pairing may be dependent on a telomeric chromosome-specific sequence. Such a model would predict that the XX pair would be subject to an obligate recombination process. This should be testable in family studies with 29Cl and another, physically linked, polymorphic sex chromosome marker.

Translocations of Yp material to the X chromosomes have been detected in a number of XX males^{39,40}. One possible mechanism which would account for this would be a rare error in the point of obligate recombination between X and Y chromosomes perhaps reflecting sequence homology proximal to the normal point of recombination. The availability of a telomeric probe for the X and Y chromosomes provides the first absolutely defined point in the human genome from which it should be possible to move to other regions of the X and Y chromosomes to test further the models postulated for X and Y chromosome exchange.

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Pseudoautosomal DNA sequences in the pairing region of the human sex chromosomes

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A DNA probe from a human Y chromosome-derived cosmid detects a single-copy genomic DNA fragment which can appear in different allelic forms shared by both sex chromosomes. Variants at this DNA locus show an autosomal pattern of inheritance, undergo recombination with sexual phenotype and can therefore be described as 'pseudoautosomal'. Another probe from the same cosmid detects a sequence repeated 15-20 times per haploid genome. These repeats also appear pseudoautosomal and map exclusively to the short-arm terminal region of each sex chromosome.

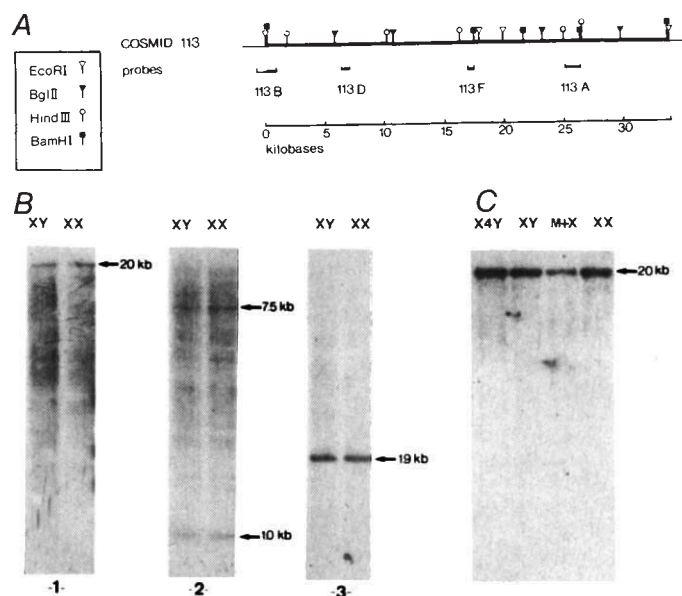
DNA SEQUENCE homologies between the mammalian X and Y chromosomes have been predicted on the basis that: (1) mammalian sex chromosomes have a common ancestral origin¹; (2) there is cytogenetic evidence for pairing and crossing-over²⁻⁵; (3) in man, non-inactivation of genes on Xp should be dosage-compensated by homologous loci on the Y ('anti-Turner' genes)^{6,7}; (4) a surface antigen, 12E7, defined by a monoclonal

antibody, encoded by a gene common to both human sex chromosomes maps to Xp and the euchromatic part of the Y chromosome⁸; (5) in mouse, there is indirect genetic evidence for crossing-over between the X and Y chromosomes⁹.

Several DNA probes from the human X and Y chromosomes have recently been isolated, some of which detect sequence homologies between both sex chromosomes. These homologies

Fig. 1 Hybridization properties of probe 113A and chromosomal assignment of *DXYS15*. **A**, Restriction endonuclease mapping of DNA probes from Y-derived cosmid 113. The upper line represents the map of cleavage sites for *Bgl*II, *Eco*RI and *Hind*III restriction enzymes. Solid bars below the restriction map indicate the localization of restriction fragments isolated and used as hybridization probes. **B**, Hybridization pattern of probe 113A to Southern blots of *Eco*RI (lane 1), *Pst*I (lane 2), and *Taq*I (lane 3) digests of human male (XY) and female (XX) DNA. **C**, Hybridization pattern of probe 113A to a Southern blot of *Eco*RI DNA digests from a human 49,XXXXY cell line (X,4Y), a normal male (XY), a normal female (XX) and HORL9X (M+X), a human-mouse hybrid containing X as the only human chromosome. Fragments detected by probe 113A are marked by an arrow.

Methods. **A**, Cosmid 113 was a cosmid from our Y-specific library^{11,19} derived from the 3E7 hybrid cell line, a mouse-human somatic hybrid containing Y as the only recognized human chromosome. Probes 113D and 113F were obtained by subcloning *Sau*3A digests of cosmid 113 in the *Bam*HI site of the M13 mp8 vector³¹. Subclone screening was performed by plaque *in situ* hybridization with the following probes: probe A, human female DNA; probe B, cosmid vector pJB8; probe C, cosmid 113. Subclones 113D and 113F were selected as hybridizing to probe C and not to probes A and B. The results obtained by *in situ* hybridization were confirmed on Southern blots of the subclones digested by *Eco*RI and *Hind*III for insert excision. Probes 113A and 113B were obtained by subcloning a *Hind*III and a *Pst*I digest respectively in the appropriate cloning sites of pBR327. Subclones were screened by *in situ* colony hybridization and by Southern blotting as described above. The inserts of subclones 113A (1,400 bp), 113B (1,800 bp), 113D (500 bp) and 113F (600 bp) were used as hybridization probes after nick-translation to a specific activity $>10^8$ c.p.m. per μ g. **B** and **C**, DNA (5–10 μ g) was completely digested with an excess of restriction endonucleases, electrophoresed on a 0.8% agarose gel, transferred to nylon membranes and baked at 80 °C for 2 h. After prehybridization at 42 °C for 4 h, in 50% formamide, 5 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 15 mM Na citrate pH 7), 5 $\times$ Denhardt's (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 20 mM Na-phosphate, pH 6.6, 50 μ g ml⁻¹ denatured salmon sperm DNA, filters were hybridized overnight at 42 °C in the same solution with addition of 10% dextran sulphate and 10–30 $\times$ 10⁶ c.p.m. of ³²P-labelled probe. Final washings of filters were in 0.1 $\times$ SSC, 0.1% SDS at 68 °C. Filters were autoradiographed with Kodak XAR5 films backed with an intensifying screen for 1–4 days at –70 °C. Accuracy of DNA dosage in each lane was monitored using a human single-copy autosomal probe.



range from a total identity at the DNA sequence level¹⁰ to a lower degree of relatedness only detected in non-stringent hybridization conditions^{11,12}. Most of the loci exhibiting strong sequence conservation also display a few restriction sites specifically linked to the X or the Y chromosome^{12,13}. This implies that homologous X/Y recombination does not occur at these DNA loci, which therefore are not 'pseudoautosomal loci' as defined by Burgoyne⁷. Indeed, those X/Y-homologous sequences that have been regionally mapped so far are not located in the putative pairing region corresponding to the distal part of the short arms of both X and Y chromosomes. Several of these sequences map to the short arm of the Y but the long arm of the X^{12,14,15} or to the short arm of the X and the long arm of the Y^{16,17}, others mapping to the long arms of both chromosomes¹⁰. In the absence of molecular evidence, even the proposed homologous pairing between the sex chromosomes in mammals has recently been questioned¹⁸. Furthermore, the most highly conserved X/Y DNA loci isolated to date have been transferred from the X to the Y chromosome during recent human evolution (ref. 13 and D. C. Page and H. J. Cooke, personal communications). However, if homologous pairing is restricted to a small part of the X and Y chromosomes, the isolation of DNA sequences from this region may require the testing of many probes. During a systematic characterization of the cosmid inserts of our Y-specific clone collection^{11,19}, we have isolated such probes defining a new category of sequences common to the X and Y chromosomes.

Pseudoautosomal DNA sequences

The probes we describe originate from cosmid 113, and their localization is indicated in Fig. 1A. When the human 49,XXXXY OX cell line was probed with 113A (Fig. 1C), we noticed a dosage effect of the hybridization signal which can be estimated as double or triple intensity compared with normal males or females. Its Y location has been confirmed by the detection of the same band in the 853-CHO hybrid (not shown), a cell line containing Y as the only cytogenetically detectable human chromosome²⁰. On the other hand, the same hybridization band

was observed in DNA digests from the HORL9X (Fig. 1C) and MOG13.9 (not shown) human-mouse cell hybrids^{21,22}, containing only the human X chromosome. No signal could be detected on control rodent DNA (shown below in Fig. 4A). Taken together, these results assign the chromosomal origin of this fragment to both the human X and Y chromosomes, and the observed dosage effect on OX DNA agrees with this location. Similar results have been obtained with probes 113B and 113D originating from different parts of the same cosmid. (Probes from cosmid 113 detecting single-copy DNA fragments define a DNA locus which is hereafter referred to as locus *DXYS15*, according to the nomenclature of the 8th Human Gene Mapping Conference (HGM8).)

Several restriction digests of normal males and females have been probed with 113A. In all cases tested, probe 113A detects identical male and female single-copy fragments, typically *Bgl*II (6.6 kilobases; kb), *Eco*RI (20 kb), *Hind*III (1.4 kb), *Pst*I (7.5 and 1 kb), *Pvu*II (10 kb), *Taq*I (1.9 kb) and *Xba*I (22 kb) fragments. The hybridization patterns of three different restriction digests (*Eco*RI, *Pst*I and *Taq*I) are shown in Fig. 1B. The restriction fragments detected by probe 113A concern 18 identical restriction sites. Similar results have been obtained with probes 113B and 113D and represent altogether more than 50 identical restriction sites. Thus, it appears that none of the fragments from locus *DXYS15* displays restriction sites occurring exclusively on only one of the sex chromosomes. This situation is reminiscent of that observed by Cooke *et al.*¹⁰, who reported complete homology between sequences present in Xq and Yq. The sequences from locus *DXYS15* differ markedly from other X/Y-homologous DNA sequences (loci *DXYS1* to *DXYS9*), which show a slight sequence divergence between the sex chromosomes^{12–14}.

With probe 113D (Fig. 1A), a restriction fragment length polymorphism (RFLP) is detected in *Taq*I digests of human males (Fig. 2A). The same allelic variations are observed in females (not shown). None of the allelic forms detected by this probe occurs exclusively on a single sex chromosome as opposed to the X- and Y-specific polymorphisms detected in loci *DXYS1*

Fig. 2 **A**, Restriction fragment length polymorphism detected by probe 113D. Hybridization pattern of probe 113D to a Southern blot of *TaqI* digests of human male DNAs. Blotting, hybridization and autoradiography were performed as indicated in Fig. 1 legend. Final washings were in $1\times$ SSC, 0.1% SDS at 68°C . **B**, Inheritance of *DXYS15* in a three-generation family. DNA samples were from kindred 66 from CEPH (Centre d'Etude du Polymorphisme Humain, Collège de France, 3 rue d'Ulm, 75005 Paris). DNA samples 01, 02, 03, 04, 06, 07, 09, 10 and 11 correspond to CEPH numbers 6601, 6602, 6603, 6604, 6606, 6607, 6609, 6610 and 6611, respectively. Southern blot of *TaqI* digests of DNAs from family 66 were probed with 113D as in **A**. For convenience, the segregating DNA fragments have been labelled *a-d*.

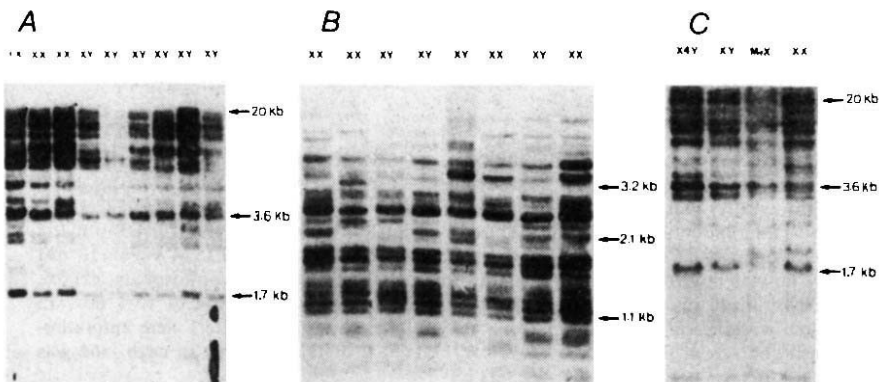
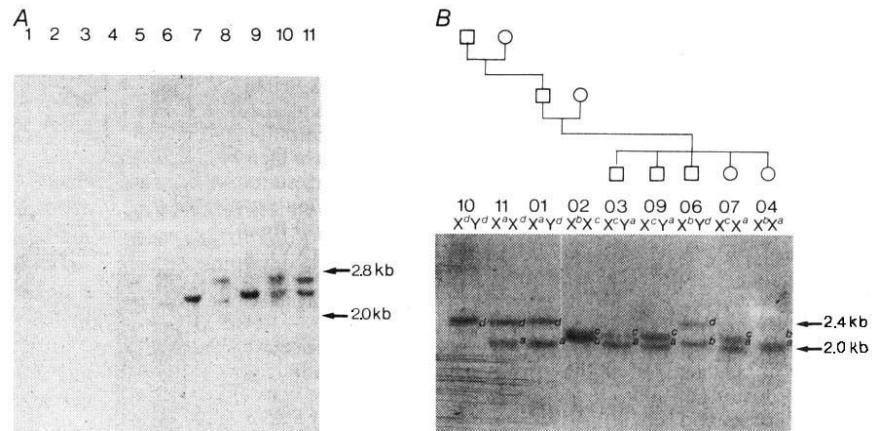


Fig. 3 Hybridization properties of probe 113F and chromosomal assignment of *DXYZ2*. Hybridization pattern of probe 113F to a Southern blot of *EcoRI* (**A**) and *TaqI* (**B**) digests of male (XY) and female (XX) DNAs. **C**, Hybridization pattern of probe 113F to a Southern blot of *EcoRI* DNA digests from a human 49,XXXXXX cell line (X,4Y), a normal male (XY), a normal female (XX) and H9L9X (M+X), a human-mouse hybrid containing X as the only human chromosome. Blotting, hybridization and autoradiography were performed as for Fig. 1. Final washings were in $0.1\times$ SSC, 0.1% SDS at 68°C . Essentially identical profiles were obtained when blots were washed in $2\times$ SSC, 0.1% SDS at 68°C .

to *DXYS9* (refs. 12–14). On the contrary, male homozygosity with a minimum of four distinct alleles can be observed in lanes 2 or 3, 4, 7 and 9 of Fig. 2A, indicating that these allelic forms are shared by both sex chromosomes. Since the RFLP detected by probe 113D has also been observed in digests using other restriction enzymes, it may be generated through an insertion/deletion mechanism. As numerous alleles have been observed, it seems that this highly variable *TaqI* fragment is prone to DNA rearrangements possibly similar to those reported recently by Jeffreys *et al.*²³. The presence of four allelic forms of one sequence with copies on both the X and Y chromosomes strongly supports ancient homology of these regions, which continue to behave as autosomal sequences (pseudoautosomal behaviour). Alternative explanations, such as identical mutation or reversion events occurring independently on each chromosome, are highly unlikely because the generation of four alleles would require three such independent, yet identical, events.

Moreover, another probe (113B) detects a *PstI* RFLP with two alleles of 3 and 10 kb (data not shown). Again, males homozygous for each allelic form have been observed, showing the occurrence of each allele on the X and Y chromosomes. This RFLP, which is not found in digests with other restriction enzymes, is probably the result of a point mutation and can be considered as a rare event which probably occurred only once either on the X or Y chromosomes. Family studies with fathers homozygous for different *TaqI* or *PstI* alleles of locus *DXYS15* could always exclude heterozygosity for a deletion, confirming that these alleles were shared by both sex chromosomes. Such polymorphic fragments represent a novel category of sequence homologies between the X and Y chromosomes and are reminiscent of the putative pseudoautosomal sequences⁷.

X/Y recombination

Family studies have confirmed the mendelian segregation of alleles for both the *TaqI* and *PstI* RFLPs. The segregation of four *TaqI* alleles is shown in the pedigree of Fig. 2B. The two paternal *TaqI* fragments are 2.4 kb (allele *d*) and 2.0 kb (allele *a*) long (case 01) whereas the two maternal *TaqI* fragments are

2.05 kb (allele *b*) and 2.15 kb (allele *c*) long (case 02). Paternal allele *d* is present in child 06 whereas all four other children (03, 09, 07 and 04) receive allele *a*. Maternal allele *c* segregates in children 03, 09 and 07, whereas children 06 and 04 inherit maternal allele *b*. Thus, each member of the progeny (children 03, 09, 06, 07 and 04) inherits one maternal and one paternal allele and each paternal or maternal allele is represented in one child at least. Two males of the progeny (03 and 09) inherit paternal allele *a*, whereas the third male (06) inherits the alternate paternal allele *d*, demonstrating that X/Y recombination can occur in male meiosis. Since the father (01) has allele *a* on his X (from his mother 11) and allele *d* on his Y (from his father 10), sons 03 and 09 with allele *a* on the Y are the X/Y recombinant progeny, while son 06 with daughters 07 and 04 are non-recombinants. The detection of two X/Y recombinants among five progeny is entirely consistent with the 50% recombination required for a pseudoautosomal sequence. However, the sample size is too small to rule out partial sex linkage.

A pseudoautosomal repeated element

Among the probes isolated from cosmid 113, we have observed a 600-base pair (bp) sequence (113F) detecting 15–20 hybridization bands in *EcoRI* DNA digests from normal males and females (Fig. 3A). Similar hybridization patterns have been observed with other restriction digests and a *TaqI* digest of several males and females is shown in Fig. 3B. It thus appears that probe 113F detects a repeated sequence (which we will refer to as *DXYZ2* according to HGM8 nomenclature) present in restriction fragments of various sizes.

In order to assess the chromosomal origin of the DNA fragments harbouring the repeated *DXYZ2* elements, we hybridized probe 113F to Southern blots of DNA digests from the 49,XXXXXX OX cell line and the X-only human-mouse hybrid cell H9L9X (Fig. 3C). The hybridization pattern obtained is essentially identical to that of Fig. 3A. In the case of OX DNA, most bands show an amplified signal indicating that most members of the *DXYZ2* family are Y-located. Furthermore, after overexposure of the autoradiogram, most of the bands observed

in male and female digests could also be detected in HORL9X, assigning the *DXYZ2* elements to the human X chromosome. Their X and Y location was confirmed by the finding that MOG13.9 and 853-CHO hybrid lines were positive when probed with 113F. (Rodent DNAs are negative; Fig. 4B, lanes 1, 18.) A few bands are irregularly present or absent in the different samples in each digest (Fig. 3A, B). They have never been found co-segregating with an autosome and may thus be ascribed to polymorphic variation. None of these variable fragments could be assigned exclusively to the X or Y chromosome, most of these elements being shared by the X and Y. It is thus most probable that a whole haploid set of the *DXYZ2* family is present on and specific for each sex chromosome.

Another element of the *DXYZ2* family, probe 708, has been isolated from a partial *Sau3A* human genomic library and is identified as a 600-bp human genomic DNA fragment containing an element highly homologous to 113F and exhibiting essentially identical hybridization properties on human genomic blots. The lack of any significant alteration in hybridization signals after washings at high ($0.1 \times \text{SSC}$, 68°C) or moderate ($2 \times \text{SSC}$, 68°C) stringency shows the high degree of sequence homology between *DXYZ2* elements.

*Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Pst*I, *Pvu*II, *Taq*I and *Xba*I restriction digests of cosmid 113 probed by 113F displayed a single hybridizing fragment, indicating that only one element of the *DXYZ2* family is present in the 36-kb insert of this cosmid. It can be inferred from the location of single-copy sequences in the close vicinity of a *DXYZ2* element that these repeats are interspersed in larger DNA regions. To estimate the frequency of *DXYZ2* elements, Southern blots of 150 independent cosmids of human origin isolated from the 3E7 somatic hybrid cell line^{11,19} were digested with *Eco*RI and hybridized to probe 708 or 113F. Among the 150 cosmids tested, three (including 113) displayed a single *Eco*RI restriction fragment hybridizing to any of the *DXYZ2* probes. Each of these three cosmids contains only one *DXYZ2* repeat. It thus appears that two consecutive *DXYZ2* elements would be separated by at least 20–40 kb (the equivalent of half a cosmid or a cosmid insert). On the other hand, 150 cosmids represent 4,000–6,000 kb, that is, 10–15% of the euchromatic part of the human Y chromosome. It is striking to observe that about 15% of *DXYZ2* copies (3 out of 20) are also present in this cosmid sampling.

Regional mapping

Regional mapping of the X-located sequences detected by probes from cosmid 113 was carried out using a panel of X-chromosome translocations segregated in somatic hybrid cells. The single-copy probe 113D detects a 20-kb human X-specific fragment exclusively in hybrid GM-194-RAG7 (Fig. 4A) in this panel. This hybrid is the only one among those tested that includes the telomeric region of the short arm (Xp22-Xpter). Other single-copy probes of cosmid 113 gave identical results.

When probed with 113F or 708, *Eco*RI digests of a more extensive panel of X-chromosome translocations isolated in hybrid cells indicated that all the *DXYZ2* elements are exclusively present in hybrids GM-194-RAG7, Cer CH S and 58 TG2-6 (Fig. 4B) and systematically absent, even after prolonged exposure, in hybrids lacking Xp22.3-Xpter. Several autosomes are present in each of the hybrids shown in Fig. 4. Absence of hybridization bands in the hybrids lacking Xp22.3-Xpter indicates that all the *DXYZ2* elements are clustered in the telomeric region of the X-chromosome short arm and that probably none maps elsewhere on the X or on an autosome. Because these results were obtained using hybridization conditions of high stringency, we cannot exclude the possibility that regions showing a lower degree of homology with the *DXYZ2* elements occur elsewhere in the genome.

The chromosomal localization of probe 708 was confirmed by *in situ* hybridization (Fig. 5). The following facts show that Yp and Xp22 were preferentially labelled. The length of Yp is 0.24% of the whole diploid genome. Thus, of the 417 grains found on all chromosomes, the expected number on Yp, assum-

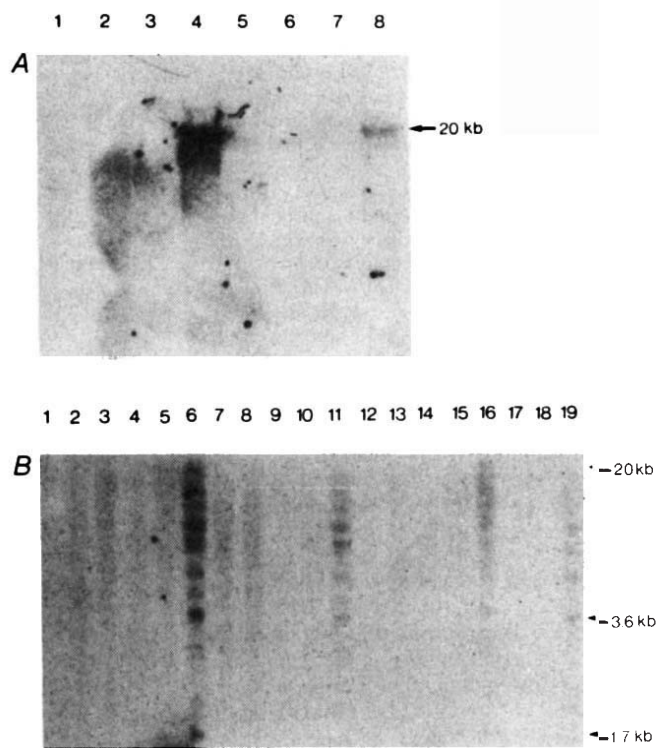


Fig. 4 X-regional localization of *DXYS15* and *DXYZ2*. **A**, Hybridization pattern of probe 113D to a Southern blot of *Eco*RI DNA digests of rodent-human hybrid cell lines. The somatic hybrids (with their human derivative X translocation products in parentheses) and control samples are as follows: lane 1, A9-HRBC2 (Xp22-Xqter); lane 2, PI-Rag7-2 (Xp22-Xqter); lane 3, G0-Rag4 (Xp11-Xqter); lane 4, human female; lane 5, Anly-Ragl (Xq12-Xqter); lane 6, A9-58 (Xq13-Xqter); lane 7, A9-GM89 (Xq24-Xqter); lane 8, GM194-Rag7 (Xpter-Xq28). **B**, Hybridization pattern of probe 113F to a Southern blot of *Eco*RI DNA digests of rodent-human hybrid cell lines. The control human and rodent digests and the somatic hybrids (with their human derivative X translocation products in parentheses) are as follows: lane 1, Chinese hamster; lane 2, CH 34X (Xp22.3-Xqter); lane 3, PI-Rag7-2 (Xp22-Xqter); lane 5, G0-Rag4 (Xp11-Xqter); lane 6, Cer CH S (Xpter-Xq11); lane 7, Cer CH H (Xq11-Xqter); lane 8, Anly-Ragl (Xq12-Xqter); lane 9, A9-58 (Xq13-Xqter); lane 10, CH 56 O (Xq21-Xqter); lane 11, 58 TG2-6 (Xpter-Xq21/22); lane 13, A9-GM89 (Xq24-Xqter); lane 14, CH 63 R (Xq26-qter); lane 15, GM97-Rag8 (Xq26-Xqter); lane 16, GM194-Rag7 (Xpter-Xq28); lane 17, A9-HRBC2 (Xp22-Xqter); lane 18, mouse; lane 19, human female.

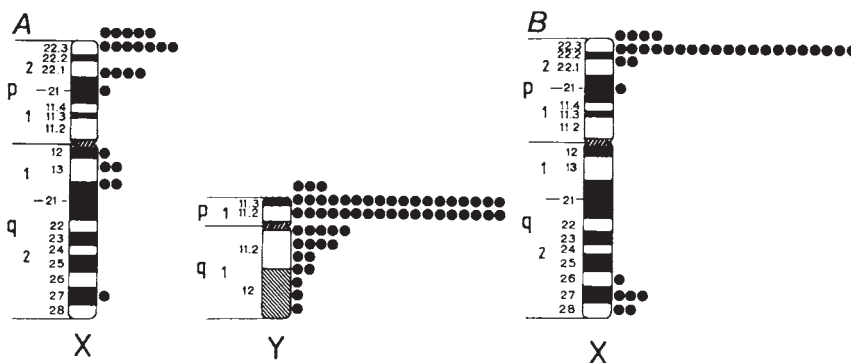
Methods. Translocations of chromosome segments of the human X were segregated on a rodent background. The resulting hybrid cell lines have been used for other X regional assignments and have been described elsewhere³². DNAs from somatic hybrid cell lines were *Eco*RI-digested and electrophoresed as indicated in Fig. 1. The digests were then transferred to dibenzylloxymethyl-cellulose filters³³. Filters were hybridized, washed and autoradiographed as for Figs 1–3.

ing random distribution, is one grain, while the observed number was 39. The length of band Xp22 is 0.33% of the diploid genome. The expected number of grains is 1, whereas 16 were found. In the experiment in which female cells were used (with two X chromosomes) the expected number of grains on band Xp22 is 3 and the number observed was 26. No autosome was preferentially labelled in these experiments.

Discussion

Our results demonstrate for the first time the existence of strictly homologous DNA sequences mapping to Yp and the telomeric region of Xp. These observations confirm most earlier predictions^{7,24} of the classical model in which X/Y synapsis was explained on the basis of DNA sequence homologies involving

Fig. 5 Cytological evidence for localization of *DXYZ2* to the regions Xp22-Xpter and Yp11.2-Ypter in a normal male and to the region Xp22-Xpter in a normal female. **A**, Schematic representation of G-banded chromosomes X and Y in a normal male, indicating the localization of grains observed on these chromosomes in 71 mitoses analysed (data not shown). Of 23 grains detected on chromosome X, 16 (70%) were localized in band Xp22 (Xp22-Xpter) and of 55 grains observed over the Y chromosome, 39 (71%) occurred in Yp11.2-Ypter. **B**, Schematic representation of G-banded chromosome X in a normal female, indicating the localization of grains observed on the two X chromosomes in 67 mitoses analysed. Of 33 grains detected on chromosome X, 26 (79%) occurred in Xp22-Xpter.



Methods. Metaphase chromosome preparations were hybridized in 50% formamide, $2 \times \text{SSCP}$, 10% dextran sulphate for 12 h at 37°C with ^3H -labelled probe 708 at a final concentration of $0.01 \mu\text{g ml}^{-1}$ and specific activity $2.5 \times 10^7 \text{ c.p.m. per } \mu\text{g DNA}$. Hybridization conditions were the same for both male and female cells. After hybridization the slides were rinsed in 50% formamide, $2 \times \text{SSC}$. The stringency at which the wash was undertaken was different in that the male cells were rinsed at 39°C and the female cells at 42°C . Slides were exposed for 5 days.

most of Yp. However, we have recently shown that at least part of the short arm of the Y chromosome is not homologous to Xp (ref. 25). The present findings might be taken to indicate that synapsis as observed by, for example, Chandley⁵ is partly between homologous and partly between non-homologous stretches of chromatids. It is most likely that each type of synapsis is characteristic of specific chromosomal regions, homologous pairing being clustered in the distal part. There is no evidence for interspersed of homologous and non-homologous regions within the Yp arm. Assuming that the Yp-specific sequences characterized to date^{12,13,24} are fully representative of the DNA sequence composition of the short arm, it seems that homologous synapsis is restricted to quite a small portion of the chromosome. The chromosomal segment characterized by the presence of *DXYZ2* elements could be the minimal length of the Xp/Yp homologous pairing region. The agreement observed between the number of *DXYZ2* repeats of our cosmid sampling and an independent estimation drawn from Southern blots, strongly suggests that most *DXYZ2* elements are interspersed. On the other hand, if our cosmid sampling, which does not include *DYZ1* and *DYZ2* repeats²⁶, is representative of the non-tandemly repeated Y-specific sequences, it would appear that *DXYZ2* elements are dispersed over at least 2% of the euchromatic part of the Y chromosome.

Two independent RFLPs were observed in the present study. In both cases the different allelic forms were equally distributed among males and females, none being especially or preferentially located on either of the sex chromosomes. As a result, these sequences can be classed as pseudoautosomal sequences according to the criteria outlined by Burgoyne⁷. By definition, pseudoautosomal sequences must recombine regularly between the X and Y chromosomes. This is actually demonstrated in the family studies we have undertaken. In the family presented, two males show recombination between the pseudoautosomal locus *DXYS15* and the sex phenotype. More recent results (F.R. and M.-C.S., unpublished) indicate that recombination involving locus *DXYS15* is a frequent event in male meiosis. This phenomenon is clearly distinct from the abnormal interchange, reminiscent of crossing-over, which occurred in the paternal meiosis of an XX male who failed to inherit his father's Xg allele but had acquired a 'Yg' allele that could only have come from his father's Y chromosome²⁷. The recombination events we observe here concern a single DNA locus. It is not known whether this recombination mechanism involves entire chromosomal regions or occurs in small areas. Family studies using several independent pseudoautosomal loci will be needed to show that the recombinations result from crossing-over and are not simply limited to a gene conversion process restricted to short DNA stretches.

Some of the *DXYZ2* repeats detected by probes 113F and 708 are polymorphic and their polymorphic variants do not

exhibit sex linkage. Regarding their chromosomal localization, *DXYZ2* elements can be considered as DNA markers of the tips of the short arms of the human sex chromosomes. It remains to be shown whether the *DXYZ2* elements are dispersed all over the region of homology or whether they are clustered in a short telomeric subregion. Similarly, it is not known if the pseudoautosomal characteristic is homogeneously distributed within the region of sequence homology or whether the more proximal homologous loci either do not recombine or recombine less than the more distal ones. In addition to the strong conservation of *DXYZ2* within the human species, hybridization to distantly related mammals such as cattle, but not to rodents, has been observed under identical stringent conditions. Such inter- and intraspecific sequence conservation suggests the possibility of some *DXYZ2*-associated functions in biological processes, for example, telomeric recognition, X non-inactivation or X/Y crossing-over.

DNA sequences homologous to the distal parts of Xp and Yp may carry genes expressed on both chromosomes. If this is so, the genes located on the non-inactivated part of the X-chromosome should be expressed in double dose in both males and females. Somatic defects associated with the 45, X karyotype seen in Turner's syndrome have been attributed to the lack of a set of the non-inactivated genes on the missing X chromosome^{6,28}. Turner stigmata are also usually found in karyotypes with deletion of Yp (Yp- females, r(Y) males)^{29,30}. Hence, the genetic deficiencies caused by the absence of a second X chromosome could be compensated in normal males by genes located on distal Yp (anti-Turner genes). It is likely that such genes have identical counterparts on distal Xp, possibly located in X/Y-homologous sequences such as those described here. The *MIC2X/MIC2Y* locus⁸ may be the first example of such genes.

With the exception of quantitative estimates, the predictions of Ferguson-Smith⁶, Polani²⁴ and Burgoyne⁷ are confirmed by our results and their implications can be approached with the new molecular tools described here.

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LETTERS TO NATURE

A new symmetrical polarization structure near the galactic centre

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Filamentary radio structures perpendicular to the plane of the Galaxy and ~ 30 pc away from the galactic centre ($l=0.2$) have been observed¹. Here we present results showing a new region of polarized emission aligned with this Continuum Arc^{1,2}. The structure of this region has the form of a central component (core) with two jet-like outer lobes. The associated total intensity maps at 4.75 and 10.7 GHz show that the core and lobes lie symmetrically with respect to arched filamentary structures lying both above and below the galactic plane—only the arches above the plane had been seen previously. The remarkable symmetry of the region suggests the location of a new 'centre of activity' within the galactic centre region at the position of the polarized core; however, total intensity maps do not distinguish this region at all except at low frequencies. A possible model involves outflow from the galactic nucleus to the vicinity of this new centre, explaining the polarization and low-frequency structure in terms of a 'cocoon' of thermal material surrounding the linear filaments seen in higher resolution studies. The regions of polarized emission then correspond to areas in which the cocoon is thinnest or absent.

The observations were performed using the 100-m radiotelescope at Effelsberg, near Bonn, between July and August 1983 (4.75-GHz data) and in July 1984 (10.7-GHz data). Both receivers (parametric amplifiers with $T_{\text{sys}}=70$ K at 4.75 GHz and FET amplifiers with $T_{\text{sys}}=100$ K at 10.7 GHz) were used in single-horn mode and had a bandwidth of 500 MHz. The half-power beamwidths were 2.4 and 1.2 arc min respectively for the two frequencies. The weather was excellent throughout the observations, enabling unswitched total power information to be used. The Stokes parameters I , U and Q were recorded simultaneously. Several calibration sources were observed at different elevations, including 3C286, 3C48, 3C147 and 3C409. These data were used to estimate the receiver characteristics and atmospheric extinction, the latter being particularly important at the higher frequency (10.7 GHz).

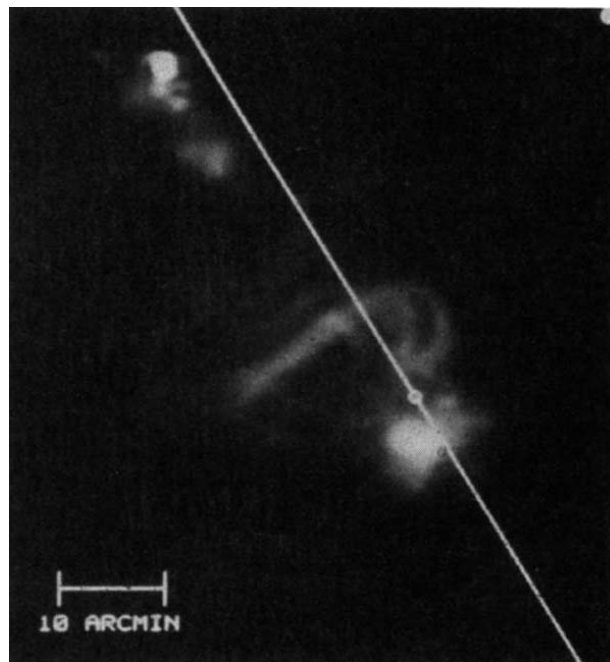


Fig. 1 Total intensity radiograph (logarithmic scale) of a $60' \times 68'$ region around the galactic centre at 10.7 GHz. The coordinates of the bottom left-hand corner are RA 17 h 45 min 28.4 s, Dec. $-29^{\circ}23'00''$. The half-power beam is shown as a filled circle at the upper right-hand corner. The position of zero galactic coordinates ($l=b=0$) is marked by a circle on the diagonal line which indicates the $b=0$ plane. The peak flux density of Sgr A is 26.8 Jy and that of Sgr B2, 17.6 Jy. The Arc reaches 2.6 Jy and the first layer towards Sgr B2, 0.7 Jy. The peak flux of the north-west arched filaments is 2.1 Jy while that for the south-east arched filaments is 0.6 Jy.

At 4.75 GHz we obtained three coverages scanned in right ascension (RA) and two in declination (Dec), covering a $40' \times 40'$ field, but with the last RA coverage being extended to $1^{\circ} \times 1^{\circ}$. The galactic centre is visible from Effelsberg for ~ 1.5 h daily, therefore for the higher resolution 10.7-GHz map the region was observed in eight independent strips, with appropriate overlap for the off-line processing and combination into one map. Because of atmospheric extinction only scans in RA (corresponding roughly to azimuth) were made. The region covered was $1^{\circ} \times 1.1^{\circ}$, the northern extension being made to include all of Sgr B2. At both frequencies the maps (made using the NOD2 procedure³) were calibrated absolutely using both calibration source information and existing lower resolution data covering a larger region. This was important for setting the edges of the maps to the correct absolute values. At 4.75 GHz we consulted

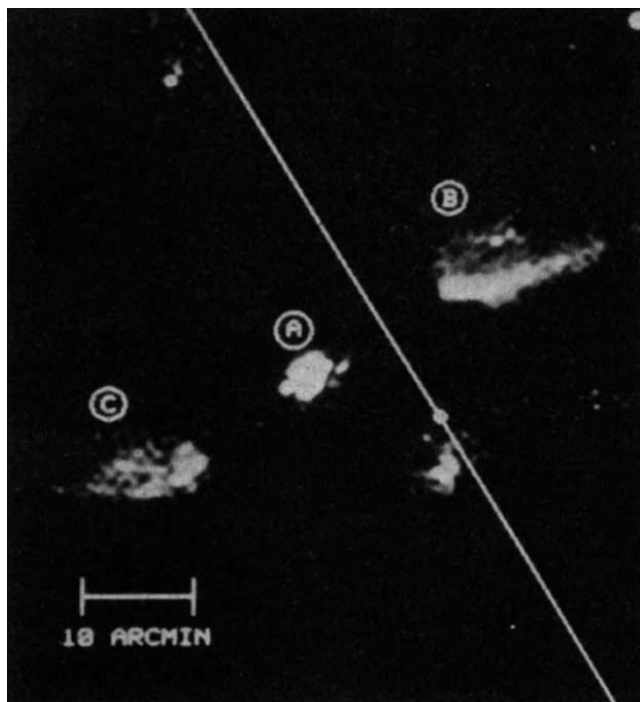


Fig. 2 Polarized intensity radiograph (linear scale) of the same region as in Fig. 1 at 10.7 GHz. The peak polarized flux density of components A, B and C is 690, 310 and 140 mJy respectively, while that for Sgr A is 260 mJy. For further details see Fig. 1.

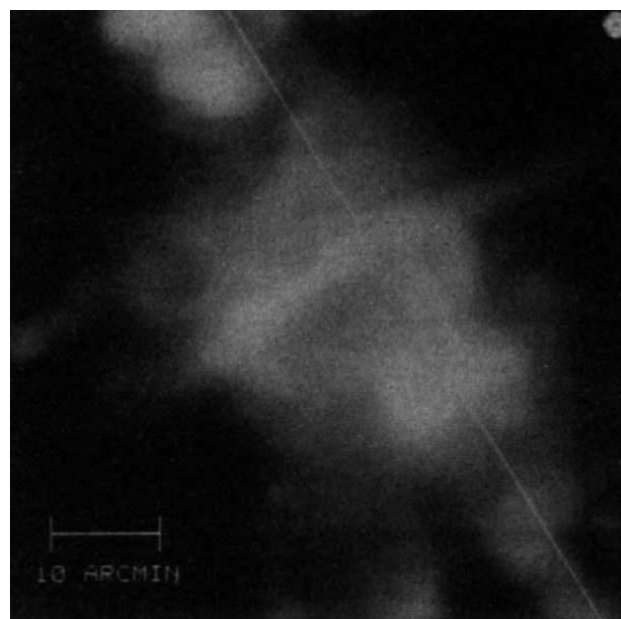


Fig. 3 Low-contrast (histogram equalized-linear scale) radio-graph of total intensity of a $60' \times 60'$ region around the galactic centre at 4.75 GHz. The bottom left-hand corner of the map coincides with the bottom left-hand corner in Fig. 1. The peak flux density for Sgr A is 58.5 Jy. The Arc reaches 6.6 Jy and the first layer towards Sgr B2, 0.9 Jy. The peak flux of the north-west arched filaments is 4.5 Jy.

the maps of Haynes *et al.*⁴ and Altenhoff *et al.*⁵ and at 10.7 GHz the edge calibration was obtained using the map of Sofue *et al.*⁶.

Details of the 4.75-GHz polarization results, derived spectral index distributions and a catalogue of discrete point sources will be presented in a subsequent paper. Here we wish to draw attention to the results of the 10.7-GHz observations and the symmetrical extensions to the Arc seen in the 4.75-GHz total intensity map. Figure 1 is a radiograph of the total intensity of 10.7-GHz emission. When this is compared with the original 20-cm Very Large Array (VLA) map of Yusef-Zadeh *et al.*¹, our greater sensitivity to low-surface-brightness extended features is immediately evident. The two arched filaments reaching from the Sgr A halo to the north-west end of the 'vertical' filaments¹ are now matched by a symmetrical pair of filaments reaching to their south-east end. In addition a fifth arched filament linking with the 'sickle' feature GO.18-0.04¹ is clearly visible. Two 'layers' of emission parallel to the Arc but displaced towards Sgr B2 can also be seen. These appear to be the brightened limbs of the helices seen by Yusef-Zadeh *et al.*¹ at higher resolution. Overall there is a remarkable symmetry in the features seen above and below the galactic plane, although not in absolute surface brightness.

Our main result is shown in Fig. 2, which is a radiograph of the intensity of polarized emission at 10.7 GHz. A core/lobe structure is visible at a position angle of 120° , parallel to the Arc. The outer lobes lie outside the extent of the Arc visible in Fig. 1 (Ref. 1) while the central core overlies not GO.18-0.04, where the true galactic plane crosses the Arc, but instead GO.16-0.13, an area of slightly enhanced continuum emission on the south-east portion of the Arc. The core A and lobes B and C have peak percentage polarizations of 22, 32 and 30% respectively in our $1.2'$ beam. The two other patches of polarization visible correspond to Sgr A and a region close to the peak of Sgr B2. The percentage values here, 1% and 3% respectively, represent the level of instrumental polarization for Sgr A, but appear to indicate some intrinsic polarization south-east of Sgr B2. In Fig. 2 the core A and north-west lobe B correspond to the sources A and B of Inoue *et al.*⁷, respectively, while the two polarization lobes reported by Tsuboi *et al.*⁸ appear to corre-

spond to our lobe B and to some combination of our sources A and C. This blending of sources A and C may be due to lower resolution and sensitivity in the Nobeyama data.

The wide frequency separation of our two observations means that we are unable to derive unambiguous rotation measures. These are now being measured for each component using the Effelsberg telescope at 5 GHz. However, if we use the average rotation measures (RM) obtained for the A and B components by Inoue *et al.*⁷, of $-1,660$ and $+800 \text{ rad m}^{-2}$ respectively, and assume that the symmetry in polarization characteristics of components B and C extends to their rotation measures, then we may estimate the intrinsic magnetic field directions from our observed E-field vectors and also estimate the differential rotation that would occur in our bandwidth. These calculations give intrinsic field directions of $+30^\circ$, $+14^\circ$ and -25° relative to the 120° position angle of the 'jet' feature seen in Fig. 2 for the components A, B and C respectively. Marginal alignment of the magnetic field direction with this feature is thus seen, particularly for the north-west lobe B, but we emphasize that detailed comparisons must await the full RM mapping being carried out at 5 GHz. (In addition, components A and C do not have unique E-field vector directions—a spread of up to 30° exists although centrally peaked at the values used above.) The predicted differential rotation across our bandwidth is 80° at 4.75 GHz and 7° at 10.7 GHz for $\text{RM} = 1,660 \text{ rad m}^{-2}$ and half these values for $\text{RM} = 800 \text{ rad m}^{-2}$. Thus, our 4.75-GHz data are significantly depolarized, particularly in position A, while the 10.7-GHz observations are relatively unaffected.

The total intensity emission corresponding to the polarized lobes B and C can be seen in lower contrast displays of the 10.7-GHz data as sharp, well-defined extensions to the Arc. The north-west extension bends slightly to the south and the south-east extension to the north at the points where the arched filaments join the Arc. This slight S-shaped morphology can be seen also in the structure of the polarization lobes (Fig. 2). The extension and the overall highly elongated bar-like structure of the Arc can be seen clearly in a low-contrast version of our 4.75-GHz map (Fig. 3). Higher contrast versions of this map are very similar (although at half the resolution) to Fig. 1 and

in particular show the two new arched filaments to the south-east and the two parallel layers on the Sgr B2 side of the Arc. The extension of the Arc to the north-west visible in Fig. 3 can also be seen in 10.5-GHz Nobeyama data filtered to remove large-scale background structure (Fig. 8 of ref. 9), and appears to indicate a smooth continuation to the large-scale ($\sim 1^\circ \times 1^\circ$) 'Sofue lobe'^{9,10} which extends to northern galactic latitude $b \sim 0.5^\circ$ before reconnecting to the plane at Sgr C. The apparent absence of a comparable lobe at negative galactic latitudes, however, and the nevertheless remarkably symmetrical structure of the polarization components A, B and C and of the Arc and its extensions as observed here, suggest that caution is required in identifying the Sofue lobe directly as a continuation of the Arc. There exists also a problem with reconciling the apparent one-dimensional geometry of the filaments¹ with the implied two-dimensional geometry of the Sofue lobe, if interpreted as a limb-brightened cylinder^{9,10}. It is more probable that the Arc and the polarization structure are phenomena causally related to the lobe, but with different emission and supporting mechanisms. What is suggested, however, is the strong possibility that similar phenomena will be seen at the point where the lobe (or western spur as seen in the Altenhoff *et al.*⁵ map at $l \sim -0.6^\circ$) reconnects with the galactic plane. A $\lambda = 20$ cm VLA map of this region¹¹ already indicates the existence of a bar-like feature at this point, perpendicular to the plane, and similar in appearance to the Arc.

The core/lobe polarization structure seen in Fig. 2 is reminiscent of the total intensity picture of a classical double radio source, and seems to argue compellingly for the core position as being a centre of activity, perhaps associated with some ejection phenomenon. Recent 160-MHz observations¹² show that the only low-frequency emission visible from the Arc is also from this position, again arguing for its special status. Early 10.7-GHz observations² mention the existence of a nonthermal source in a position very close to the core (GO.16-0.15) where recombination lines were absent. However, recent total intensity pictures of this region at high frequencies (our Fig. 1 or Fig. 1 of ref. 1), do not distinguish the region at all—it appears to be a smoothly connected part of the filamentary structure. This is a major obstacle to any interpretation involving ejection from this position as the explanation of the Arc or the polarization lobes.

To get around this, we may extend the suggestion put forward in ref. 12. The structure seen in polarization and low-frequency emission may be the result of obscuration by a non-uniform screen of thermal material, threaded by the ambient magnetic field, which attenuates at low frequencies and depolarizes the underlying assumed synchrotron radiation. (That the underlying radiation is synchrotron seems certain from the high percentage polarizations, 30% in the outer lobes and on smaller scales in component A¹², and from the extreme linear geometry of the filaments, suggesting a magnetic field as the underlying organizing agent.) Some support for this suggestion comes from the observation that component A lies in a large gap symmetrically placed with respect to the arched filaments (Fig. 1). Thus if material were being fed along the arched filaments from the vicinity of the galactic nucleus as part of a supply of matter to the envelope surrounding the vertical filaments, it might be the case that only close to the termination point of the filaments on the Arc would sufficient material accumulate to flatten the spectral index and depolarize the underlying synchrotron radiation. The amount of material required for this purpose is estimated in ref. 12 at a few tens to a few hundreds of solar masses, although the suggestion is made that the material originates from the ambient gas surrounding the vertical filaments¹³, rather than from Sgr A itself. Against this explanation, however, is the circular appearance of the central component A and the two small blobs of polarized emission either side of it pointing to the lobes (Fig. 2). This would seem difficult to generate via non-uniformities in an intervening screen without artificial assumptions as to the screen's geometry. Higher resolution polarization observations¹² show that component A is rather

less ordered than suggested by its appearance in our observations, so this objection may be irrelevant. Also, the symmetry of B and C extends only to general appearance and percentage polarization and not to their detailed morphology so that again an explanation in terms of the effects of screening material is not ruled out. The general symmetry evident might in any case be expected on the basis of an approximate symmetry in distribution of screening material with respect to the galactic plane.

The nature of the polarization structure and its central component is so far unclear, as is its stability and lifetime. (It does not, unlike the Arc itself, lie along a surface of constant angular velocity in the Oort mass distribution¹⁴.) However, it is clear that it must be reconciled with any general picture of activity at the galactic centre. Such a picture would seem now to have to include a 'chimney' of buoyant outflowing material (the Sofue lobe) similar to that seen in optical emission as a spur in the Crab nebula¹⁵ and in radio emission as one-sided lobes in other spiral galaxies¹⁶. The appearance of the Arc feature and the polarization structure at the point where the chimney crosses the galactic plane, will certainly prove to be important in understanding the complexities of the galactic centre.

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Origin of the galactic centre lobes

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Recent observations of the 10-GHz continuum¹ have indicated that there is a pair of lobes having ridges of intensity extending from the galactic centre region in a direction perpendicular to the galactic plane. This observation has attracted considerable attention as it is clearly an indication of dynamic processes occurring at the centre of our Galaxy that are similar to those occurring in the nuclei of radio galaxies, though different in scale and in strength². Here we interpret these lobes as being due to a magnetodynamic acceleration mechanism in which the production and relaxation of the magnetic twist induced by the rotation of the contracting gas disk play a part. The plasma is accelerated in a conical cylinder with a helical velocity field, reproducing the observed feature of radio lobes.

The structure (referred to as GCL, for galactic centre lobes) that is located on both sides of Sgr A with some asymmetry, has proved to have further interesting features. Yusef-Zadeh *et al.*³ showed by means of high-resolution Very Large Array (VLA) observations that a source related to the galactic centre has a very peculiar structure. The structure, called the galactic centre radio arc (GCRA), is embedded in the galactic dust and has a

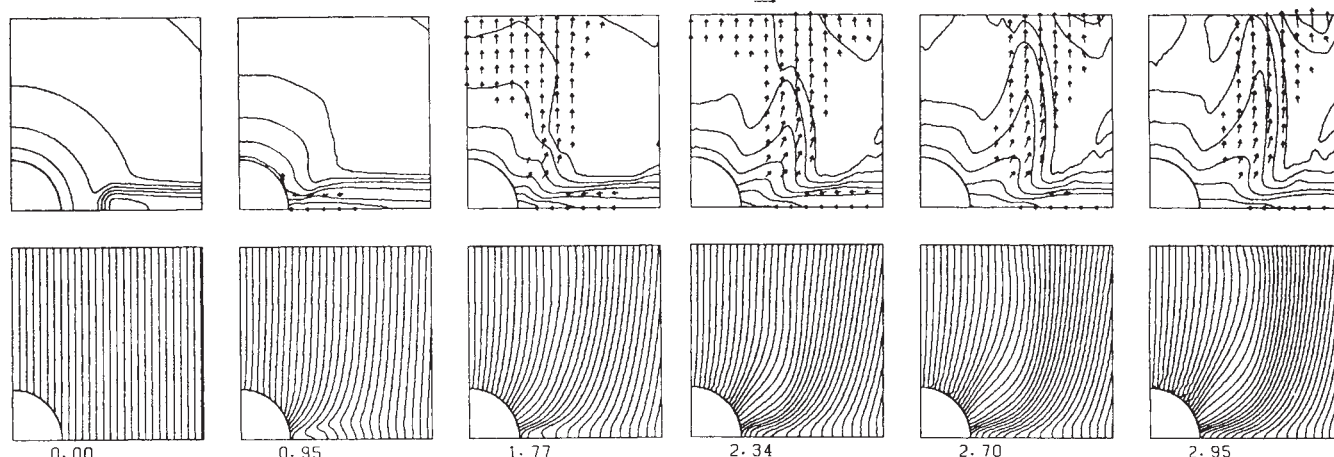


Fig. 1 Evolution with time of: upper sequence, the poloidal velocity vector, $v_{\parallel}$ ($= v_r, v_z$), overlaid on the density contour; lower sequence, the poloidal magnetic field lines, $B_{\parallel}$ ($= B_r, B_z$). The numbers below each figure represent units of τ . It can be seen that a hollow cylindrical flow of denser material just like GCL is formed as the magnetic twist accumulated by the rotation of the disk starts relaxing toward the z -direction. The circle at the centre indicates the inner free boundary for the calculation.

thread-like appearance, strongly suggestive of magnetic fields, with one part lying vertically just at the foot, and in the extension, of the eastern ridge of GCL. The other part connects the north end of this vertical thread-like structure to Sgr A. Recent VLA observations⁴ show that a similar structure is found at the foot of the western ridge of GCL. By measuring the multi-band polarization of the eastern ridge at 10 GHz, Inoue *et al.*⁵ found that the polarization has peaks at both ends of the vertical part of the GCRA, and the sense of Faraday rotation is opposite at the north and south ends. A further measurement of Faraday rotation-corrected polarization (M. Tsuboi and M. Inoue, personal communication) reveals that the line-of-sight magnetic field component along the eastern ridge of GCL is directed towards us on its northern arm and away from us on its southern arm. The direction of the projected component of the magnetic field is parallel to the ridge of GCL, and the degree of linear polarization is as high as several tens of per cent. These observations suggest strongly that the entire process which led to the ejection of GCL is related to the magnetic field.

As for the formation of double radio lobes associated with radio galaxies and quasistellar objects, it has been suggested that the magnetic field and rotation might have important roles^{6,7}. More recent theoretical studies have attributed the formation of these lobes to the guiding of the strong wind from their source by the funnel structure in the thick disk surrounding the source of these objects⁸⁻¹¹, with a few noting the importance of the magnetic field in the formation of jets¹²⁻¹⁵. For our Galaxy, however, before the discovery of the GCL it was believed that the activity, if it existed, was too weak to produce such phenomena.

Here we propose a magnetodynamic mechanism which explains the production of GCL by the combined effect of the rotating disk and of the magnetic field near the galactic centre. The mechanism relies on the continual production of a magnetic twist by the rotation of the disk around the galactic centre; the unequilibrated magnetic pressure gradient built up near the surface of the disk in this process causes the relaxation of the magnetic twist to the $\pm z$ directions, pushing out the plasma with helical velocity. We describe in the following the method used and the results obtained.

Consider a rotating accretion disk which entwines a part of the large-scale magnetic field. A system of ideal magneto-hydrodynamic equations, in a pseudo-three-dimensional formulation, for a cylindrical symmetry ($\partial/\partial\phi=0$, but allowing ϕ -components of vector quantities) is solved numerically using a modified Lax-Wendroff scheme. A set of non-dimensional parameters (R_1 , R_2 and R_3) specifies the physical situation of the

problem under consideration, where $R_1 \equiv v_s^2/v_K^2$, $R_2 \equiv V_A^2/v_K^2$ and $R_3 \equiv v_{\text{rot}}^2/v_K^2$, and $v_s \equiv (\gamma RT)^{1/2}$, $V_A \equiv B/(4\pi\rho)^{1/2}$, $v_K \equiv (GM/r)^{1/2}$, the R_i s representing the relative importance of the pressure gradient force, the Lorentz force, and the centrifugal force with respect to the gravity force, respectively. (v_{rot} , rotation velocity; v_K , keplerian velocity). A similar solution may hold if the set $(R_1, R_2, R_3)_0$ at a point near the inner edge of the disk, as well as the initial distributions of quantities in the relative coordinate, r/L , (where r is the radius and L is the typical scale of the problem) are the same. The timescale differs from case to case as $\tau = L/v_K$.

Values of R_i are determined for the galactic centre region as follows: Mezger and Pauls¹⁶ have demonstrated the existence of an extended cloud of H II with a thickness and diameter of ~ 100 pc and 300 pc, respectively, a mean ionized gas density $\rho \sim 10 m_H \text{ cm}^{-3}$ (m_H is hydrogen mass), and an electron temperature of 5,000 K. The total mass involved in the part of the disk with $r \leq 60$ pc (mean distance of the east and west ridges of the lobes) from the galactic centre (Sgr A) is $\sim 3 \times 10^8 M_\odot$, and the rotation velocity at $r = 60$ pc is $\sim 180 \text{ km s}^{-1}$, as derived from the H I and CO line observations of the galactic centre region¹⁷. The magnetic field strength may be roughly evaluated by applying the equipartition law for energy densities of high-energy electrons and the magnetic field; using our 10-GHz radio continuum data for the galactic centre region², we obtain $B \sim 2 \times 10^{-5} \text{ G}$ at the location of the GCRA. From these quantities we obtain $v_s = 8 \text{ km s}^{-1}$, $V_A = 14 \text{ km s}^{-1}$, in the disk near the GCRA. These quantities yield the set of parameters $(R_1, R_2, R_3)_0 \sim (1.8 \times 10^{-3}, 6.1 \times 10^{-3}, 1 - \delta)$ where δ is a small fraction of unity, assuming that the part of the disk under consideration is rotating at a sub-keplerian velocity and is still in the contracting stage. It is interesting that the set of non-dimensional parameters thus derived is not very different from that for a corresponding point ($3.0 \times 10^{-3}, 7.2 \times 10^{-3}, 1 - \delta$) in the protostellar case examined previously¹⁵. Thus, we may expect that the outflows from the galactic centre region will be similar (though very different in scale) to those in the star-forming regions, which, in our view, also result from the magnetic field becoming twisted up in the rotational motion of the contracting accretion disk towards the end of the star-formation process.

Figures 1 and 2 show the result of numerical simulations for the present situation. The calculation is preliminary because we assumed that the effective gravity source is a point mass at the centre. Cases corresponding to a more realistic situation in the galactic centre region are now being dealt with, and a fuller discussion of such cases, which confirm the basic validity of the present result, will be published elsewhere¹⁸.

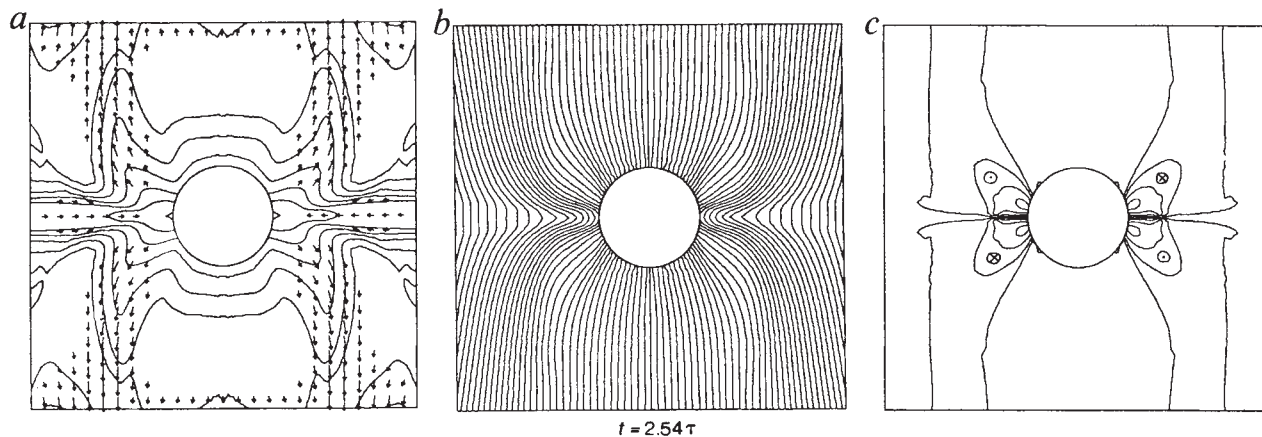


Fig. 2 *a*, The poloidal velocity vector overlain on the density contour. *b*, The poloidal magnetic field lines. *c*, The contour of the toroidal magnetic field strength, B_ϕ . (All parameters at $t = 2.54\tau$.) It can be seen in *b* that the poloidal magnetic field has a vertical component in the disk, together with some other field lines obliquely connected to the inner part, corresponding to the vertical and oblique structures of the GCRA. From *c*, it can be seen that the toroidal component has opposite polarities on both sides of the equatorial plane, as Inoue *et al.*'s observation indicates⁵.

Figure 1 shows the time development after a disk is formed in a plane perpendicular to the large-scale magnetic field, and is still contracting ($R_3 < 0$). The circle at the centre is the inner free boundary for the calculation. The upper sequence (overlay of the velocity field projected on the density contour in the r - z plane) shows that, following the preceding fast-mode front due to the squeezing around the axis of rotation, there appears a denser flow rising with a horn-shaped profile, corresponding to a hollow conical cylinder in three dimensions. Figure 2 shows the situation at $t = 2.54\tau$ ($\tau \equiv L/v_K$ at $r = L$) in full, as an example. The poloidal magnetic field $B_\parallel (=B_r, B_z)$ is pulled both inwards and into the ϕ -direction by the contracting rotation of the disk, and the toroidal field B_ϕ is produced continually. The accumulated B_ϕ relaxes along $B_\parallel$ as a magnetic-twist front. The signs of B_ϕ above and below the plane of the disk are opposite because it is produced by the rotating disk winding up the poloidal field. Clearly, the energy of the cylindrical flow derives from the rotational energy of the dense part of the disk, mediated by the action of the magnetic field. The unequilibrated gradient in B_ϕ , which is continually produced by the effect of rotation, pushes up the mass of the less dense (but dense enough to make high contrast in the halo) surficial region of the disk into a conical jet.

It is readily noted from Figs 1 and 2 that the density contour has a similarity to GCL observed by Sofue and Handa¹, and that the near-disk configuration of $B_\parallel$ and the distribution of B_ϕ may explain the shape of the GCRA and the reversal of the line-of-sight component of the magnetic field^{5,6}. It is characteristic of our mechanism that the velocity field has v_ϕ (though not shown here) which, together with $v_\parallel$, yields a helical velocity field. The velocity of the outflow is of the order of 150–200 km s⁻¹, and the rotational velocity near the disk and near the front is of the same order as this, but is considerably less in between. Preliminary results from the observation of GCL in CO lines, now being done using the Nagoya University 4m mm-wave telescope (I. Suwa, Y. Fukui, Y. S. and T. Handa, personal communication) suggest that the observed velocity field is not inconsistent with our model.

We require some support for our assumptions, for example, the assumption that the initial magnetic field is perpendicular to the plane of the disk, and $R_3 \equiv v_{\text{rot}}^2(r)/v_K^2 < 1$. The former assumption, which seems to be observationally actually the case with the star-forming regions¹⁹, is likely to be relevant in a wide variety of condensations of the magnetized mass. This is because it is easier for the initial condensation to occur along B_0 than perpendicular to it²⁰, and also because the angular momentum component perpendicular to the magnetic field in the initial

cloud tends to be damped in the earlier phase of contraction, and the component parallel to B_0 remains^{21,22}. Although the magnetic field in the galactic arms is largely aligned with the arm structure, it is possible that the magnetic field in the galactic centre part has poloidal components. The observations in refs 3, 5 and by Tsuboi *et al.* (personal communication) strongly support this notion.

The second assumption—that $R_3 < 1$ —is obviously the case if the accreted gas is still outside its Kepler radius. Even after it reaches the Kepler radius, however, the condition $R_3 < 1$ may be brought about in the final phase of accretion due to the loss of angular momentum by the production of the magnetic twist and the mass ejection by the present mechanism. The rotation is thus braked ($R_3 < 1$), and the disk mass can continue to fall spirally toward the centre of gravity¹⁸. A larger amount of energy is released and can be converted to that of the bipolar jet as the centre is approached, since the mediator, the magnetic field, gains strength in the compression and winding up operates more effectively.

Finally, note that the process discussed here may be either a continuous process or a train of discrete events, depending on the supply of magnetized material. Similarly, the process can be asymmetric with respect to the axis or the equatorial plane, if there is such an asymmetry in the distribution of the density or magnetic field in the accreted cloud, although the calculation was performed by assuming axisymmetry as an idealization.

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Probing the local interstellar medium

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Kurth *et al.*¹, using Voyager radio data and a simple model of solar-wind expansion, have inferred the location of the heliospheric shock to be 46 AU. Additional consideration of their data and the use of a modern model of the distant solar wind allow us to reduce this estimate to as little as 30 AU. The shock occurs where the solar wind pressure falls to that of the local interstellar medium (LISM). This value for the distance to the shock is less than half that usually expected, indicating that the parameters thought to describe the LISM differ from those commonly assumed. Provisionally accepting this interpretation, we applied a sophisticated model of solar-wind expansion to deduce a range of parameters for the LISM that predicts a comparable shock location. Specifically, we find that either the interstellar magnetic field is more than double the presently accepted value of 0.3 nT, or the pressure due to galactic cosmic rays with energies near 0.1 MeV is that obtained by simple extrapolation of the observed flux at higher energies inside the heliosphere, or some combination of these two external effects to yield an effective interstellar pressure approximately quadruple present estimates.

As the solar wind flows into the LISM, it undergoes a transition to subsonic flow and then expands until it is dispersed within the LISM. Using Voyager data, Kurth *et al.*¹ reported on 2–3-kHz radio emission at 18 AU, which they suggest is radiation at the second harmonic of the plasma frequency at the heliospheric shock (erroneously called the heliopause²). They assume that the solar-wind electron concentration falls off as r^{-2} , the terminating shock is a strong shock with a fourfold density enhancement, and the lower cutoff of the observed emission is the local plasma frequency at the spacecraft. They then infer the heliocentric distance to the heliospheric shock in the direction of motion of the Solar System through the LISM (the upstream or apex direction) to be $r_s = 46$ AU. Accepting this interpretation of the 3-kHz radio emission, we refine the estimate for r_s and place new limits on properties of the LISM. We can do this because r_s depends on the total hydromagnetic pressure that the LISM exerts on the heliosphere and is thus a function of LISM parameters.

Before leaving the interpretation of the Voyager data, we point out one possible deficiency in the analysis. The 2-kHz cutoff and an inverse square variation of density with radius imply a density of $1.6 \times 10^7 \text{ m}^{-3}$ at 1 AU, more than double the observed average value. The cutoff could result from the spacecraft being shielded by a high-density co-rotating interaction region rather than being immersed in a uniformly dense solar wind, so this is not an objection to the general ideas presented by Kurth *et al.* *In situ* values quoted by Kurth *et al.* show that the local solar wind had a lower density than implied by the 2-kHz cutoff. The significance of this point is that more reasonable values for the solar wind number density require that the heliospheric shock be located not at 46 AU, but as close as 30 AU. This value is still not precluded by interplanetary data. Pioneer 10 is nearly at that distance but in the antapex direction.

Commonly accepted parameters for the LISM and the heliosphere are summarized in Table 1 (refs 3–9). We have used both the data from early 1983 and a knowledge of solar cycle variations for solar wind values^{4,5} to arrive at a set of appropriate heliospheric parameters for the observation interval. These numbers are used to calculate what can be called the standard value for r_s in which cosmic ray and interstellar neutral gas pressures are ignored, and the solar wind mass density falls as $1/r^2$. The result is a discrepancy between theory and experiment; r_s is predicted to lie at 117 AU—far beyond the 46 AU distance inferred by Kurth *et al.*

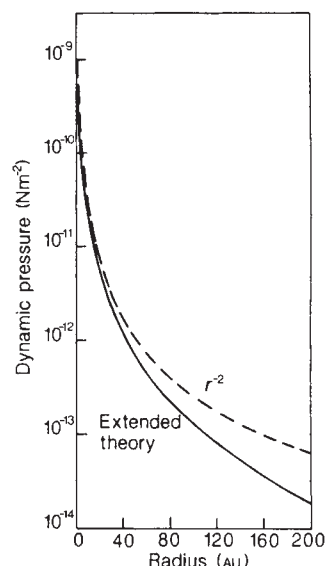


Fig. 1 Variation of the solar wind dynamic pressure with radius, using the heliospheric parameters from Table 1. The solid line represents the variation when the effects of magneto hydrodynamic meridional transport^{8,9} and interstellar neutrals^{2,12} are included. The broken line represents the variation without these effects, resulting in an r^{-2} dependence.

The difference between the standard value for r_s and the observed value is reduced by including in the solar-wind flow model both meridional transport (which moves mass polewards from the Equator)^{10,11} and mass loading by charge-exchange with interstellar neutrals^{3,6,8,9}. The meridional transport causes the density to fall off as $r^{-2} (1 - 0.065 \ln r)$ and the charge exchange causes the velocity to decrease approximately as $u_e (1 - 0.0028 r)$ while maintaining the mass flux to be a constant independent of radius, where r is expressed in AU. The dynamic pressure exerted by the solar wind on the LISM, in the presence of these two effects and using the parameters from Table 1, is shown in Fig. 1. In the absence of meridional transport and interstellar neutrals, the dynamic pressure falls off as r^{-2} . Meridional transport reduces the standard estimate of r_s to 98 AU, and charge exchange with interstellar neutrals further reduces it to 86 AU. Nevertheless, the standard estimate is still far beyond the inferred value of 46 AU. To reduce the distance to the heliospheric shock to that inferred by Kurth *et al.*, it is necessary to alter some parameter of the LISM or to introduce

Table 1 Interstellar and heliospheric parameters

Interstellar parameters	
n_g (ion and neutral concentration)	Standard values $4 \times 10^4 \text{ m}^{-3}$
T_g (plasma and neutral temperature)	10^4 K
$\langle m_g \rangle$ (average ion and neutral mass)	Proton mass (m_p)
V_g (flow speed of ions and neutrals relative to the Solar System)	$22.5 \times 10^4 \text{ m s}^{-1}$ (in the ecliptic and approximately counter to the direction of Voyager 1 motion)
B_g (interstellar magnetic field)	0.3 nT
α (compression factor of B_g^2 around the heliosphere)	2.25
Heliospheric parameters	
μ_0 (solar wind flow speed)	$4.5 \times 10^5 \text{ m s}^{-1}$
n_0 (1 AU)	$6.5 \times 10^6 \text{ m}^{-3}$
B_0 (1 AU)	7 nT
K (multiplies total interstellar pressure to account for dynamics in subsonic flow regime)	1.483 (assuming B_0 is 7 nT)
$\langle m_0 \rangle$ (average ion mass)	$1.16 \times m_p$

a physical process not yet considered in the calculation of the standard value. Two possibilities for altering the LISM are apparent.

First, the interstellar magnetic field strength, B_g —by far the least well determined parameter—can be adjusted to reduce r_s to the required value. The interstellar magnetic field is pushed aside by and draped over the heliospheric cavity so that it is generally more nearly parallel than perpendicular to the heliospheric boundary, and the magnetic field strength is increased by a factor of ~ 1.5 through this deformation and compression^{8,9}. The direction of the unperturbed interstellar magnetic field relative to the heliopause boundary in the apex direction is, however, completely unknown, so we only use the 1.5 enhancement factor as an estimate of the interstellar magnetic field effect. Setting the solar-wind pressure at 46 AU equal to $B_g^2/2\mu_0$, we find $B_g = 0.73$ nT. This is more than double the value quoted in Table 1, which is larger than permitted by observational constraints.

The second possibility is that low-energy galactic cosmic rays exert a significant pressure on the solar wind^{3,6,12}. The subsonic solar wind beyond the heliospheric shock shields the inner heliosphere from cosmic rays with energies below about 5 MeV. Below ~ 1 MeV, this process causes the galactic cosmic ray flux to fall nearly to zero inside the heliospheric shock and represents a force on the solar wind that can be approximated by an additional pressure equivalent to the interstellar thermal pressure. The flux of cosmic rays outside the heliospheric shock with energies < 1 MeV is not known, but if it is larger than within the heliosphere, the excess flux outside exerts this pressure.

The integral flux of cosmic rays above 0.1 MeV required to produce a pressure of 4×10^{-13} N m⁻² is 5×10^4 protons m⁻² s⁻¹ sr⁻¹. This is the flux that would be obtained by simply extrapolating the integral spectrum observed within the heliospheric shock above 10 MeV (where the effect of the solar wind is negligible) to 0.1 MeV using an E^{-1} power law^{13,14}. This is a relatively small extrapolation and it thus appears that cosmic-ray pressure on the heliopause may be responsible for bringing the theoretical estimate of r_s to the distance suggested by observation.

Returning to the estimate of r_s using the Voyager radio data, there is the question of whether r_s should be 46 AU or 30 AU. If 30 AU is the preferred value, our results for the interstellar magnetic field strength and cosmic ray flux must be suitably modified. To bring the theoretical value of r_s in to 30 AU, we use Fig. 1 to find that the lower limit for B_g would be 1.2 nT, or the flux of cosmic rays above 0.1 MeV would increase to 1.5×10^5 protons m⁻² s⁻¹ sr⁻¹. Finally, we note that the physical relevance of our analysis rests on the validity of the interpretation by Kurth *et al.*¹ of the single observational set of radio emissions obtained by Voyager during a 6-month interval starting in late 1983. The phenomenon has not been reproduced (W. R. Kurth, personal communication), which raises the possibility of an alternative interpretation to that of the detection of the heliospheric shock.

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Osmium isotope constraints on Earth's late accretionary history

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Osmium isotope measurements reported by Allègre and Luck^{1,2} indicate that terrestrial osmiridiums evolved in a mantle source region in which the osmium/rhenium ratio falls strictly within the range found in chondrites. This suggests that the highly siderophile elements in the Earth's mantle were introduced by a late influx of chondritic material and are not a result of endogenous processes. I have now examined the available data in more detail and conclude that the inferred Os/Re ratio of the Earth's mantle matches the E group and C3 chondrites, but that C1 and probably C2 chondrites were not major components of the material accreted in the late stages of mantle formation.

The abundances in the Earth's upper mantle of such highly siderophile elements as the platinum group elements (PGE; Ru, Rh, Pd, Os, Ir, Pt), Re and Au are much higher than would be expected if the Earth's Fe–Ni core had been formed by a low-pressure equilibrium process^{3–6}. Recent studies of spinel lherzolite xenoliths of upper mantle origin have shown that the worldwide distribution of PGE is surprisingly uniform, averaging 3.4 parts per 10⁹ (p.p.b.) Os, 3.7 p.p.b. Ir and 4.6 p.p.b. Pd^{7–9}; apparently the presence of small amounts of sulphide 'buffers' the PGE abundances over a wide range of lithophile element depletion¹⁰. In addition, and perhaps more importantly, the PGE are present in approximately cosmic (that is, C1 chondrite) proportions. Abundances of Au and Re are more variable and the variability seems to be correlated with depletion of lithophile elements. The fact that the higher values of Au and Re in the least depleted xenoliths approach cosmic proportions with respect to the PGE strongly suggests that the concentration range observed in spinel lherzolites is the result of depletion from an initially chondrite-like pattern equivalent to about 0.01 of C1 chondrite abundances. It is now widely accepted that the highly siderophile elements were probably introduced by a late influx of siderophile-rich chondritic material after formation of the Earth's core^{5,7,9,11,12}. By analogy with the formation of the major lunar basins, the highly siderophile complement of the Earth's upper mantle above the 670-km discontinuity may have been contributed by the influx of 10²⁵ g of planetesimals during the first 600 Myr of the Earth's history. For a reasonable range of geocentric planetesimal velocity, and making allowance for gravitational focusing, this influx rate corresponds closely to that inferred for the Moon⁷. There are dissenting views, however. Brett¹³ argues that low-pressure (~ 30 kbar) equilibration with an Fe–S–O eutectic liquid may reproduce the observed mantle siderophile abundances (particularly the moderately siderophile elements Ni and Co, which are not discussed here). Similarly, Jones and Drake¹⁴ suggest that the highly siderophile element content of the upper mantle is the result of a small amount of residual metal remaining after core separation. The important point is that the 'exogenous' or late-influx model requires originally chondritic siderophile element ratios whereas 'endogenous' models such as those cited above must explain why chondritic ratios are observed. Thus, observations suggesting strictly chondritic siderophile ratios simultaneously strengthen exogenous and undermine endogenous models.

Recent developments in the mass spectrometry of Os and Re have enabled Luck, Allègre and Birck^{1,2,15} to repeat with very high precision the pioneering work of Herr and co-workers^{16–18}. Isotope analyses of iron meteorites and the metallic phase of chondrites describe a linear array on a plot of ¹⁸⁷Os/¹⁸⁶Os against ¹⁸⁷Re/¹⁸⁶Os. Regression analysis yields an initial ¹⁸⁷Os/¹⁸⁶Os = 0.805 ± 0.006 and a decay constant, λ , of ¹⁸⁷Re = $(1.52 \pm 0.04) \times 10^{-11}$ yr⁻¹, corresponding to a half-life of 4.56×10^{10} yr assuming that chondrites and iron meteorites have a common age of 4.55×10^9 yr (ref. 1).

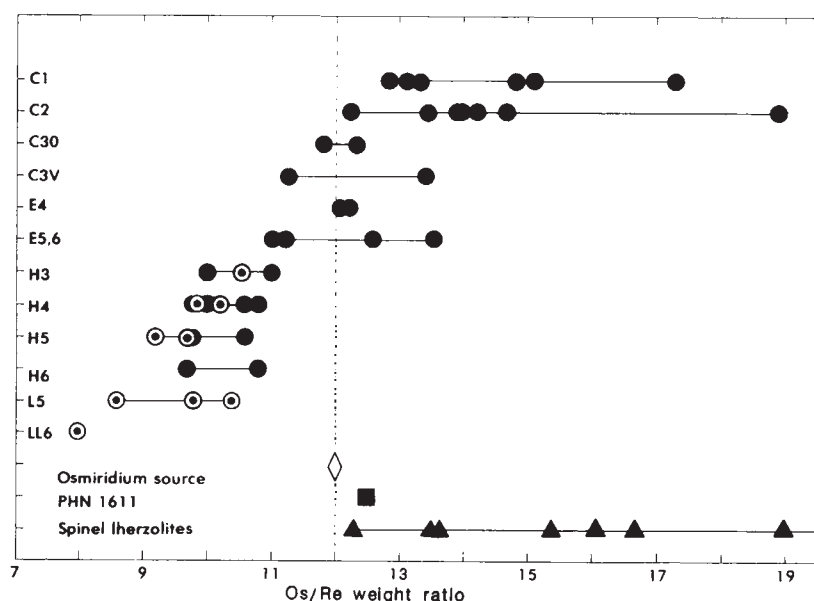


Fig. 1 Comparison of Os/Re ratios in chondrite groups with that of the Earth's upper mantle. Closed symbols, RNAA; open symbols, MSID.

Of particular relevance to the question of highly siderophile elements in the mantle is the determination of $^{187}\text{Os}/^{186}\text{Os}$ ratios in osmiridium samples of known provenance and age². The mineral osmiridium is composed largely of Os and Ir, and contains no Re. As a result, the $^{187}\text{Os}/^{186}\text{Os}$ in osmiridium is identical to that developed in the mantle source region until the time, t , of separation and emplacement. By making the approximation $\lambda t = (e^{\lambda t} - 1)$ and plotting $(^{187}\text{Os}/^{186}\text{Os})$ against t , 5 osmiridiums from placer deposits of known provenance and age in Australia, South Africa, Columbia and the Soviet Union closely fit a straight line ($r=0.998$) with an intercept at age 4.55×10^9 yr of $(^{187}\text{Os}/^{186}\text{Os})_{\text{initial}} = 0.805$, an identical value to that obtained for meteorites^{1,2}. Consequently, the mantle evolution curve would be unchanged if the initial meteorite $^{187}\text{Os}/^{186}\text{Os}$ ratio were used as an additional constraint, as in the work of Luck and Allègre^{1,2}. The slope gives a value for $(^{187}\text{Re}/^{186}\text{Os})_{\text{mantle}}$ of 3.39, which corresponds to a mass ratio Os/Re of 11.8. Since this ratio lies in the middle of the chondritic range (as pointed out in ref. 2, and discussed in more detail below), it confirms the previous inference that Re in the mantle was originally in chondritic proportion to Os, and by extension, to the other highly siderophile elements that have chondritic ratios relative to Os. Because Os and Re are refractory siderophiles and are not greatly fractionated by cosmochemical processes, this is a highly predictable result if one accepts the exogenous model^{5-7,9}. By the same token, a high degree of coincidence must be invoked for endogenous models^{13,14}.

The striking precision of the mantle Os/Re ratio derived from osmiridium makes it tempting to carry the analysis a stage further. Although the lack of variability of the Os/Re ratio in meteorites is often stressed, in detail there appear to be significant differences between meteorite groups. For example, in the meteorite ^{187}Re - ^{187}Os isochron, points for chondritic metal lie marginally above the iron meteorite line^{1,15}. Considerable recent radiochemical neutron activation (RNAA) data now exist for Re and Os abundances in chondrites which appear to show quite regular variations in Os/Re ratio with meteorite grouping^{1,2,19-23}. These whole chondrite data appear to give Os/Re ratios directly comparable to mass spectrometric isotope dilution (MSID) values for chondritic metal, respective RNAA and MSID values being 10.0 and 9.9 for the Beaver Creek (group H4) meteorite, 9.8 and 10.2 for the Menow (H4) meteorite, and 9.7 and 9.7 for the Ambapur Nagla (H5) meteorite.

Since small differences in the Os/Re ratio may be informative, the osmiridium regression analysis has been repeated

using the expression

$$\left(\frac{^{187}\text{Os}}{^{186}\text{Os}}\right)_t = \left(\frac{^{187}\text{Os}}{^{186}\text{Os}}\right)_0 - \left(\frac{^{187}\text{Re}}{^{186}\text{Os}}\right)_0 (e^{\lambda t} - 1)$$

where λ is the ^{187}Re decay constant, t is the age of the osmiridium and the subscripts refer respectively to the ratios at time t and time zero (that is, now). To improve the estimate, $(^{187}\text{Os}/^{186}\text{Os})_t$ was plotted against $(e^{\lambda t} - 1)$ instead of the λt approximation, to yield $(^{187}\text{Re}/^{186}\text{Os})_{\text{mantle}} = 3.32$, corresponding to Os/Re = 12.06 by weight. The difference between $e^{\lambda t} - 1$ and λt is small ($\sim 3.5\%$ for $t = 4.55 \times 10^9$ yr) and may be comparable to uncertainties in the ages of osmiridium emplacement. Nevertheless, the age error is probably random, and substitution of $e^{\lambda t} - 1$ for λt removes a small but significant bias from the estimate.

If this value truly approximates the primordial mantle ratio, comparison with the chondrite value suggests that only the C30, C3V and E group chondrites are candidates for the material accreted in the late stages by the Earth (Fig. 1). C1 chondrites do not match the mantle ratio. Without invoking special fractionation effects, the concept of an Earth composed entirely of C1 chondrites seems implausible¹¹. On the other hand, the isotope data tend to support the suggestion, based on rare gas abundances, that the late addition of material resembled C3V chondrites in composition²⁴. The isotopically derived Os/Re mantle ratio closely resembles that of the sheared garnet lherzolite PHN 1611, whose pyrolite-like composition suggests that it may represent our best approximation to undepleted mantle material²³. The spinel lherzolites, as suggested above, approach the true mantle value in the least depleted samples.

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Evidence for lower crustal ductile strain localization in southern New York

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Historic triangulation data have been analysed to determine whether intraplate seismicity is associated with ongoing ductile deformation in the lower crust. The model we have attempted to test is basically analogous to strain accumulation and release along plate-boundary strike-slip faults like the San Andreas Fault in California. That is, beneath an elastic-seismogenic upper crust ~20 km thick, strain is preferentially localized within ductile shear zones in the lower crust due to broad-scale plate driving forces. The localized lower-crustal ductile strain causes stress and strain to accumulate elastically in the brittle crust which is eventually released in crustal earthquakes. At greater depths, this localized shear deformation probably develops into pervasive ductile flow. Numerous geodetic measurements along the San Andreas Fault confirm that earthquakes in the brittle upper crust are produced by the release of elastic strain that results from ongoing ductile shear or slip in the lower crust^{1,2}. We have found evidence of high rates of crustal deformation in southern New York which suggest that localized ductile shear is occurring in the lower crust.

The presence of ductile shear zones in the lower crust has been suggested by workers who have studied geological structures within exhumed shear zones³⁻⁶ and effects of temperature and pressure on the dominant deformation mechanisms of crustal rocks⁷. Laboratory studies suggest several mechanisms that may be responsible for the development of such zones in lower crustal conditions⁸. Several authors have suggested that significant deformation may occur along discrete large-scale shear zones within continents^{9,10}, and play a key role in continental fragmentation¹¹. In the context of the model tested here, these shear zones would be sites of localized ductile shear in the intraplate lower crust.

We have analysed repeated triangulation measurements made by the National Geodetic Survey (NGS) in three areas: the region of the Ramapo fault zone of northeastern New Jersey and south-east New York, the New Madrid area of southeastern Missouri and northeastern Arkansas, and the Charleston, South Carolina area. Large earthquakes occurred near Charleston in 1886 and near New Madrid in 1811-12. All three areas are currently seismically active.

To establish horizontal control points for map makers, engineers and others, NGS routinely measures the angles in networks of control points. The primary purpose of making the measurements is not the detection of crustal motion and the angles are not systematically reobserved. However, the demands for additional control often require that areas be resurveyed and in the process some angles are reobserved. Only the shear strain

components of the strain tensor can be determined from repeated angle observations.

While insufficient repeated angles were available to obtain useful results in the New Madrid and Charleston zones, 130 repeated angle measurements were available in the region of the Ramapo fault zone. For the purpose of analysis, we split these angles into five separate groups (Fig. 1). One group of angles in New York and Connecticut is near the northeastern end of the broad zone of contemporary seismicity associated with the Ramapo fault system. These angles were measured at various times between 1872 and 1973. Outside the seismicity zone, 100 repeated angles were measured between 1885 and 1969; 31 angles in Eastern Long Island, 27 angles in Western Long Island, 26 angles near the eastern and western boundaries of New Jersey, and 15 angles in eastern Pennsylvania.

The deviatoric part of the strain field can be described by two average strain rate parameters, $\dot{\gamma}_1$, $\dot{\gamma}_2$, which define shear strain rates on NW-SE and E-W striking planes, respectively. The change in an observed angle over a particular time period can be written as

$$\Delta\alpha = \Delta t \cdot [(\sin 2\theta_2 - \sin 2\theta_1)\dot{\gamma}_1 + (\cos 2\theta_2 - \cos 2\theta_1)\dot{\gamma}_2]$$

where $\Delta\alpha$ is the observed change in angle, Δt is the time between the two observations, and θ_1 and θ_2 are the azimuths of the two sides of the angle¹². $\dot{\gamma}_1$ and $\dot{\gamma}_2$ were estimated from the observed angle changes by a least-squares process¹³. An equivalent way of presenting the strain field is in terms of the azimuth of the plane across which shear is maximum

$$\Psi = \begin{cases} \frac{1}{2} \tan^{-1}(\dot{\gamma}_1/\dot{\gamma}_2) + 90^\circ, & \text{if } \dot{\gamma}_1 < 0 \text{ and } \dot{\gamma}_2 > 0 \\ \frac{1}{2} \tan^{-1}(\dot{\gamma}_1/\dot{\gamma}_2) & \text{otherwise} \end{cases}$$

and the shear strain rate across that plane

$$\dot{\gamma} = (\dot{\gamma}_1^2 + \dot{\gamma}_2^2)^{1/2}$$

As shown in Table 1 and Fig. 1, the groups of angles in Pennsylvania, New Jersey, and Eastern Long Island showed no statistically significant strain rate at the 95% (2σ) confidence level. However, the Western Long Island and New York-Connecticut groups of angles both show statistically significant strain, with maximum strain occurring on NNW-SSE and N-S striking planes, respectively.

In the Western Long Island and New York-Connecticut data sets, the strain rates are surprisingly quite large, roughly equivalent to strain rates observed near the San Andreas fault². The critical question is: how realistic are the estimates of standard deviations quoted in Table 1 and Fig. 1 because if the quoted standard deviations are correct, then it is unlikely that the observed strains result from errors. The original observations of most of the angles used in this study were third order surveys. These angles have errors that are normally distributed with a standard deviation of 1.2 s (ref. 14). The later surveys were mostly first or second order with standard deviations of 0.6-0.8 s (ref. 14). The strain rate parameters were calculated from a weighted least squares adjustment. The weights used for the observations were obtained from the NGS data file and varied from 2.78 s^{-2} to 0.6 s^{-2} corresponding to standard errors in the observations of 0.6 s to 1.3 s. The standard deviations given in Table 1 and Fig. 2 were obtained by propagating the error estimates of the observations through the matrix equation used to calculate $\dot{\gamma}_1$ and $\dot{\gamma}_2$. The error estimates are not dependent on the fit of the observations to the time-and-space-uniform model. The *a posteriori* standard deviation of an observation of *a priori* unit weight does depend on the fit of the observations to the model. This quantity is

$$\sigma_0 = \left[\frac{\sum (o_i - c_i)^2}{n_{d.o.f.}} \right]^{1/2}$$

where o_i is the observed *i*th angle, c_i is the calculated *i*th angle, σ_i is the *a priori* standard error of the *i*th angle, and $n_{d.o.f.}$ is the number of degrees of freedom.

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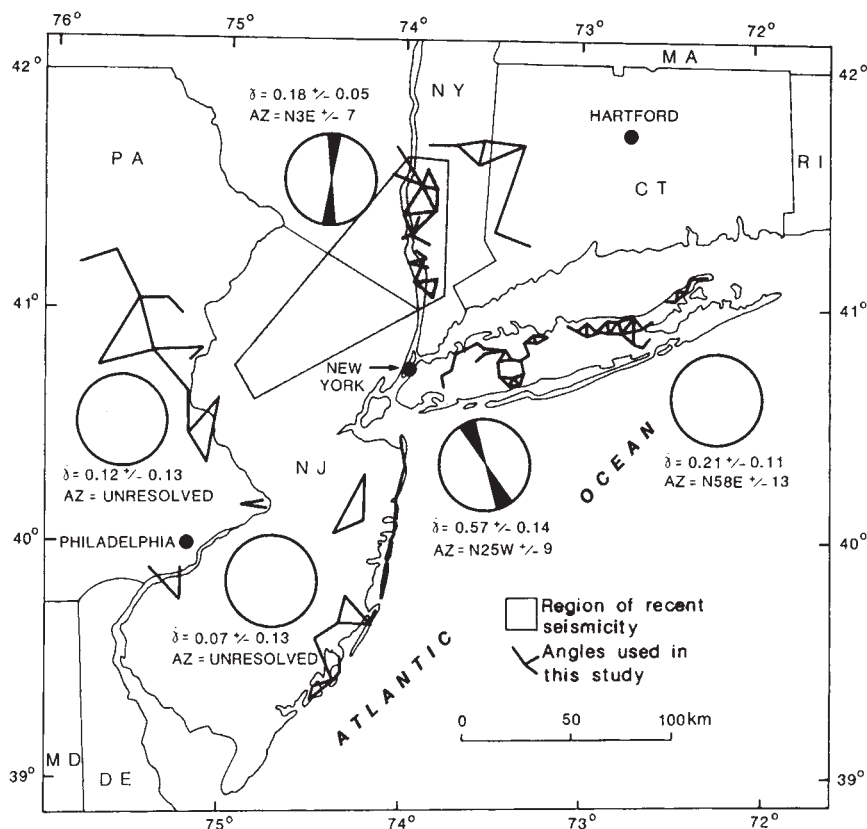


Fig. 1 Area of study in the New York-Connecticut-New Jersey-Pennsylvania area. The box indicates the zone of contemporary seismicity associated with the Ramapo fault zone. The angles analysed in this study were divided into the five groups shown which are described in the text and summarized in Table 1. The symbols and rates indicate the orientation (degrees clockwise from north) the magnitude (μ strain/year) of maximum right-lateral shear strain rate.

If the observed model is a correct description of the deformation and if the *a priori* standard deviations accurately describe the scatter in the observations then σ_0 will have a value of 1. For the five data sets in Table 1, σ_0 is 1.4, 0.7, 1.3, 1.3 and 1.2. We conclude that the *a priori* error distribution of the observations is reasonable and consequently that the standard deviations for the strain components are reasonable.

To test further that the computed strain rates in the New York-Connecticut and Western Long Island areas were not due to undetected measurement errors or blunders in the data, each group of angles was split into two independent subsets. In both New York-Connecticut and Western Long Island, the subsets yielded strain rates quite similar to that of the group as a whole (Table 2). Consequently, although the results indicate surprising large strain rates, we can find no basis for rejecting them; they appear to be real.

The cause of the anomalous strain rates observed in the New York-Connecticut and Western Long Island areas is not clear. Although there is widespread low-magnitude seismicity in this area (Fig. 2), no earthquakes of sufficient magnitude have occurred during historic time which could account for the observed strain as coseismic deformation¹⁵. Thus, the most likely explanation of the observed strain rates is in terms of localized ductile

deformation in the lower crust.

The most common model used to interpret observed strain near the San Andreas Fault¹⁶ is one in which a vertical strike-slip fault is locked to some depth, D , and slipping at a constant rate, $\dot{b}$, at depth greater than D . Then the shear strain rate of the surface at a distance x from the fault trace is given by

$$\dot{\gamma}(x) = \frac{\pi \dot{b}}{D} \frac{1}{[1 + (x/D)]^2}$$

If such a model describes the deformation reported here, deep-crustal aseismic slip would be occurring in the lower crust at rates of several centimetres per year. Such rates could probably not be sustained in the lower crust for more than several hundred years without a major earthquake occurring.

While ductile straining in the lower crust could be quite complex these data indicate evidence of high rates of strain at depth regardless of any specific model. If a simple strike-slip model is correct, the planes of maximum shear strain strike north to north-northeast whereas the mapped faults and other surficial geological structures in the area generally strike more easterly¹⁷. This would imply either that the surficial features are not directly related to the zone of ductile strain localization at depth or that the actual deformation at depth is much more

Table 1 Strain rate parameters on five groups separated according to the number of angles used in the analysis

Area	No. of angles	Dates of observations	$\dot{\gamma}_1$ ($\mu\text{rad yr}^{-1}$)	$\dot{\gamma}_2$ ($\mu\text{rad yr}^{-1}$)	$\dot{\gamma}$ ($\mu\text{rad yr}^{-1}$)	Azimuth Ψ
New York-Connecticut	30	1862-1973	-0.02 ± 0.04	-0.18 ± 0.05	0.18 ± 0.05	N3° E $\pm$ 7
Western Long Island	27	1932-1967	0.43 ± 0.15	-0.37 ± 0.15	0.57 ± 0.14	N25° W $\pm$ 9
Eastern Long Island	31	1939-1967	-0.19 ± 0.11	0.09 ± 0.09	0.21 ± 0.11	N58° E $\pm$ 13
Northern New Jersey	26	1931-1962	-0.05 ± 0.16	0.04 ± 0.14	0.07 ± 0.13	N68° E $\pm$ 74
Eastern Pennsylvania	15	1885-1969	-0.06 ± 0.20	0.10 ± 0.18	0.12 ± 0.13	N74° E $\pm$ 56

$\dot{\gamma}_1$, right-lateral shear strain rate on a NW-SE striking plane⁻¹, uncertainty is 1 s.d. $\dot{\gamma}_2$, Right-lateral shear strain rate on an E-W striking plane⁻¹. $\dot{\gamma}$, Maximum shear strain rate. Azimuth, orientation of plane of maximum shear measured clockwise from the north.

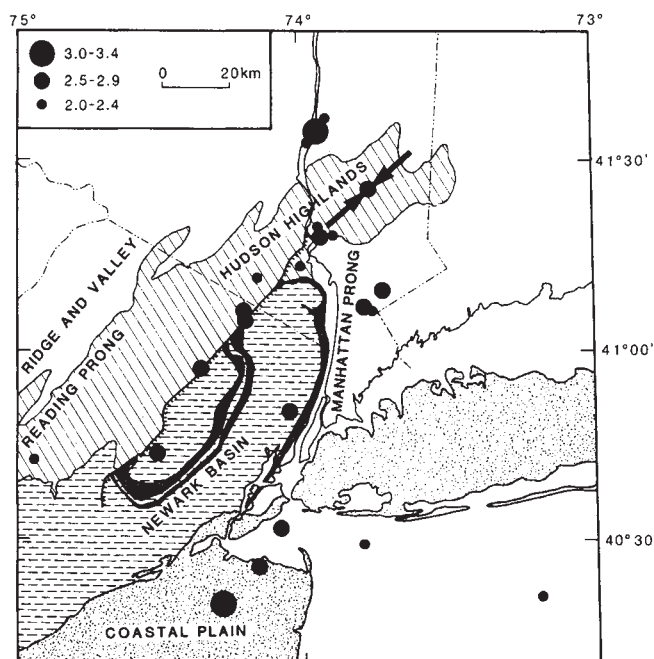


Fig. 2 Generalized geological map¹⁷ showing location of micro-earthquakes recorded by the Lamont-Doherty Geological Observatory from 1974 to 1981 (ref. 34). The arrows indicate the direction of maximum horizontal compression determined from recent *in situ* measurements in the Hudson Highlands²⁰.

complicated than represented by such a simple model. In the latter case, the true nature of strain at depth may be obscured by our ability to estimate only the shear components of the strain field.

Several lines of evidence may support the contention that lower crustal aseismic deformation could be occurring along N–S to NNW–SSE planes in this area. The NE–SW direction of maximum horizontal compression that is implied by the shear deformation is quite reasonable for this area. It is consistent with the direction defined by numerous stress data in western New York^{18,19}. Recent stress measurements within the area covered by the New York–Connecticut data²⁰ indicate NE–SW compression (Fig. 2), as does a reanalysis of focal mechanisms in New England²¹. Also, a N–S striking trend of seismicity runs through the area of the New York–Connecticut data¹⁵ and recent microearthquakes near the Hudson river ~30 km north of New York City suggest that earthquakes in the area align along N–S striking planes in response to NE–SW to ENE–SWS compression²². The largest historic earthquake in this area was a widely-felt event with an estimated Modified Mercalli intensity of VII in 1884.

The most surprising aspect of these results is the extremely high strain rate observed. If aseismic displacements were occur-

Table 2 Strain rates of subsets of groups identified in Table 1

Subset	No. of angles	Dates of observations	γ	Ψ
New York–Connecticut	12	1862–1973	0.19 ± 0.05	$N2^\circ E \pm 8$
New York–Connecticut	18	1932–1966	0.17 ± 0.09	$N7^\circ E \pm 15$
West Long Island	8	1864–1948	0.62 ± 0.23	$N22^\circ W \pm 11$
West Long Island	19	1932–1967	0.60 ± 0.19	$N18^\circ W \pm 11$

ring in the lower crust they would have to be of the order of several centimetres per year to explain the data. These rates seem quite fast for an intraplate area. As derived from studies of global plate motion²³, the North American Plate is estimated to be moving only $\sim 2.7 \text{ cm yr}^{-1}$ in the vicinity of the Ramapo fault zone. It is not clear how so much internal deformation could occur within the plate for extended geological time periods. Thus, one possibility is that, in a geological sense, we are observing a relatively short-term strain episode.

One of the most striking aspects of intraplate seismic zones is their apparent association with zones of ancient tectonism^{9,11,24–27}. This association has led to the widely-held presumption that large intraplate earthquakes tend to occur in zones of crustal weakness (that is, zones of anomalously low-strength in the seismogenic, upper 20 km, of the brittle crust). There are several problems with this hypothesis, however. First, no adequate physical explanation has been put forth to explain which specific fault zones in the brittle crust would be expected to have low strength. Laboratory studies show that, with the exception of several clay minerals not stable at great depth, the frictional strength of nearly all rocks and minerals are about the same²⁸. Available *in situ* stress measurements supports the relevance of such data for active faults²⁹. Second, in the areas near intraplate seismic zones there is no evidence of the anomalous stress field that would be expected if the seismicity were due to stress concentrations around 'weak' intrusive rocks of low elastic stiffness³⁰. In fact, the opposite seems true—intraplate earthquakes seem to occur in response to a broad, regionally uniform stress field³¹. Finally, it is not clear how 'weak' zones in the upper crust would be capable of accumulating sufficient elastic strain energy to produce major earthquakes, or how, once released, strain energy would reaccumulate in such zones rapidly enough to explain observed recurrence times on the order of hundreds to thousands of years^{32,33}. Because it is not possible to answer such fundamental questions, the 'zone of crustal weakness' hypothesis has been essentially useless in distinguishing which ancient tectonic zones might be expected to produce large damaging earthquakes in the future. The most important characteristic of the model presented here is that sites of large intraplate earthquakes are controlled by zones of localized strain in the lower crust which concentrate stress elastically in the upper crust. Identification of these zones might indicate sites where large intraplate earthquakes could occur in the future.

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Low- M_r hydrocarbons generated during hydrous and dry pyrolysis of kerogen

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The validity of applying laboratory pyrolysis experiments to simulating the maturation of organic matter in sedimentary basins has been vigorously debated¹⁹. We report here results from the generation of hydrocarbons of low relative molecular mass (M_r) in both hydrous and dry pyrolysis. A principal difference is that under dry conditions in the presence of montmorillonite, catalysis occurs with respect to generation of low- M_r hydrocarbons¹⁰ but no such effect is evident for hydrous conditions, probably because of a reduction in the clay's acidity. In addition, olefins which were previously reported as not being present in the products of hydrous pyrolyses^{4,6,11} were found to be produced in the C_2 - C_6 range in comparable amounts under both hydrous and dry pyrolyses at 300 °C and may form in the course of kerogen catagenesis in nature but disappear with geologic time due to their instability. These studies have relevance to understanding the interactions between kerogen and minerals in sedimentary rocks and to processes in the formation of natural gas.

Green River Formation kerogen was used for both the hydrous and dry pyrolyses¹⁰. The procedure for isolation of the kerogen, mixing it with different minerals, the pyrolysis technique and analysis of the products after pyrolysis is described elsewhere¹⁰. After 5 ml of distilled water was added to a Pyrex tube containing 0.5 g of the Green River kerogen or 10.5 g of kerogen-mineral mixture (0.5 g kerogen + 10 g mineral), the water was frozen in dry ice/acetone mixture and the tubes were sealed under vacuum. The tubes were then inserted into a 1-litre Parr bomb that was filled with distilled water in order to maintain equal fluid pressure on both sides of the glass walls. The tubes were heated at 200 °C, 300 °C and 400 °C for varying times under dry conditions and under hydrous conditions at 300 °C for 2, 10, and 100 hours. Corrosion of the glass walls occurred especially in the tubes that contained clay minerals, resulting in loss of the gaseous fraction produced after 100 hours of heating and in all experiments attempted at 400 °C. Compounds in the C_1 - C_6

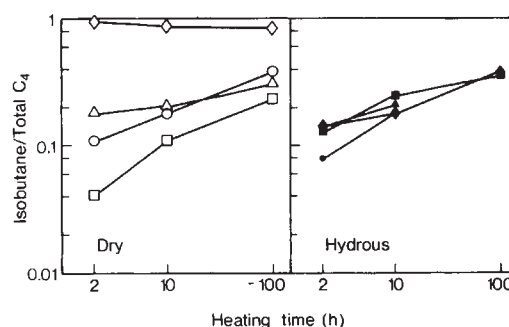


Fig. 1 Comparison between isobutane/total C_4 ratios of hydrocarbons generated under dry and hydrous pyrolyses. Circles denote results for kerogen heated to 300 °C alone; squares, kerogen with calcite, triangles kerogen + illite and diamonds kerogen with montmorillonite.

range were identified and quantified by gas chromatography and gas chromatography/mass spectrometry¹⁰.

The quantities of volatile hydrocarbons produced by hydrous pyrolysis of the Green River kerogen are reported in Table 1. The results of the dry condition are reported and discussed elsewhere¹⁰. The major products of pyrolyses are polar bitumens and a lesser amount of pyrobitumen¹². Two major differences between the dry and hydrous experiments are apparent. First, lower quantities of C_1 - C_6 hydrocarbons are generated in the hydrous compared to the dry pyrolyses. Second, only under dry condition, significantly larger amounts of C_4 - C_6 hydrocarbons are produced in the presence of montmorillonite than by kerogen alone. The lack of significant difference between the amounts of hydrocarbon gases and condensates produced in the presence and absence of montmorillonite, indicates the lack of catalysis by clays in the presence of excess water.

Dominance of branched hydrocarbons in the C_4 - C_6 range has been shown to be an important indicator for catalytic cracking of hydrocarbons via carbonium-ion (carbocation) intermediates^{10,13,14}. This dominance is depicted by the high ratio of isobutane/total C_4 hydrocarbons generated from the Green River kerogen under dry pyrolysis condition (Fig. 1), in the presence of montmorillonite. This higher ratio for kerogen heated with montmorillonite is not apparent for the products of hydrous pyrolysis (Fig. 1). The absence of catalytic effect of montmorillonite, both on the types and quantities of light hydrocarbons produced, is probably due to the decrease of the clay's

Table 1 Amounts (mg per g kerogen) of C_1 - C_6 hydrocarbons generated from pyrolysis of Green River kerogen under hydrous condition at 300 °C

	C ₆																	Total C ₁ -C ₆
	C ₁	C ₂		C ₃		C ₄ ‡			C ₅ §				Normal	Tot. branc. sat.	Tot. unsat.	Cyc.		
		Unsat.†	Sat.	Unsat.	Sat.	Normal	Iso	Tot. unsat.	Normal	Iso.	Tot. unsat.	Cyc.						
2 hours																		
K*	0.31	0.043	0.033	0.013	0.043	0.008	0.009	0.10	0.007	0.006	0.024	—	0.29	0.029	0.007	0.031	0.96	
I	0.32	0.038	0.037	0.006	0.044	0.009	0.009	0.045	0.005	0.005	0.015	—	0.22	0.028	0.003	0.033	0.81	
M	0.63	0.055	0.050	0.030	0.098	0.030	0.015	0.068	0.010	0.009	0.040	—	0.24	0.027	0.029	0.033	1.4	
C	0.31	0.033	0.037	0.011	0.051	0.008	0.010	0.058	0.010	0.008	0.019	—	0.14	0.026	0.004	0.027	0.75	
10 hours																		
K	1.6	0.16	0.28	0.18	0.38	0.13	0.17	0.63	0.10	0.094	0.34	0.004	0.73	0.15	0.24	0.080	5.3	
I	1.4	0.12	0.24	0.11	0.29	0.072	0.094	0.29	0.062	0.058	0.21	0.001	0.40	0.11	0.14	0.065	3.7	
M	1.3	0.093	0.16	0.084	0.21	0.058	0.064	0.24	0.034	0.036	0.13	—	0.15	0.052	0.077	0.028	2.7	
C	0.74	0.052	0.11	0.037	0.13	0.024	0.043	0.10	0.016	0.023	0.049	—	0.071	0.028	0.022	0.015	1.4	
100 hours																		
K	2.2	0.14	0.80	0.27	0.86	0.32	0.48	0.50	0.27	0.32	0.52	0.042	0.51	0.51	0.49	0.12	8.3	
C	3.5	0.18	1.0	0.31	0.97	0.34	0.51	0.57	0.26	0.33	0.54	0.024	0.30	0.48	0.39	0.11	9.9	

For further details see Table 2 in ref. 10.

* K, kerogen; I, kerogen + illite; M, kerogen + montmorillonite; C, kerogen + calcite.

† Sat., unsat., branc., cyc.: saturated, unsaturated, branched and cyclic hydrocarbons, respectively; tot, total; tr, traces.

‡ C_4 iso: 2-methylpropane; C_4 tot unsat: 1-butene, 2-butene.

§ C_5 iso: 2-methylbutane; C_5 tot unsat: 3-methyl-1-butene, 1-pentene, 2-methyl-1-butene, 2-pentene, 2-methyl-2-butene, pentadiene; C_5 cyc: cyclopentane.

|| C_6 tot branc sat: 2,2-dimethylbutane, 2,3-dimethylbutane, 2-methylpentane, 3-methylpentane; C_6 tot unsat: 4-methyl-1-pentene, unsaturated C_6 , 2-methyl-1-pentene, 2 or 3 hexene; C_6 tot cyc: methylcyclopentane, cyclohexane.

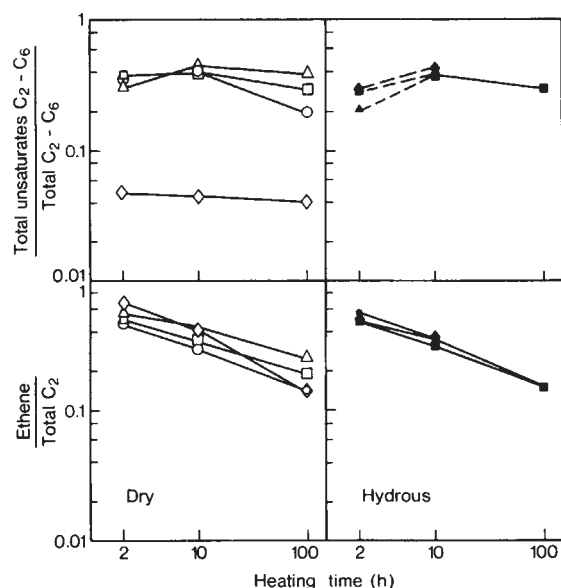


Fig. 2 Comparison between ratios of olefins/total hydrocarbons generated under dry and hydrous pyrolyses. Symbols as for Fig. 1.

acidity resulting from excess water. At low acidity values only few protons are available to initiate hydrocarbon cracking via carbonium-ion mechanism. The result is thermal hydrocarbon cracking via a free-radical mechanism in which isomerization is not preferred. Applying these results to natural environments, where clay-rich source rocks (especially rocks containing significant quantities of smectite) in the zone of oil and gas generation contain low amounts of water, the acidity of the clays could permit catalytic processes to occur. There are indications from sedimentary basins that catalytic processes involving generation of light hydrocarbons do occur^{15,16}.

Figure 2 shows the ratio of olefins/total hydrocarbons for C_2 and total C_2 - C_6 hydrocarbons. The olefins constitute an important component of low molecular weight hydrocarbons produced during pyrolysis under dry conditions, especially for the experiments where kerogen was pyrolysed alone or with calcite and illite, where 30–45% of the products in the C_2 - C_6 range are olefins (Fig. 2). Significantly lower ratios of olefins/total hydrocarbons are apparent for the corresponding products in the presence of montmorillonite (approximately 4% olefins, Fig. 2). It is also apparent from Fig. 2 that the relative quantities of olefins produced in the hydrous pyrolyses are similar to those formed in the dry experiments (except for pyrolysis in the presence of montmorillonite under dry condition).

One of the main compositional differences between laboratory pyrolyses products and hydrocarbons generated in nature is the formation of significant amounts of olefins in the former, whereas they are rare in the latter. Some studies have shown that no olefins were formed under water saturated conditions (hydrous pyrolysis), and the authors suggest that such conditions better simulate the processes in nature^{4,6,11}. However, the olefins searched for in these studies are in the carbon number range of C_{12} and higher. In our experiment, at mild heating temperatures (200–300 °C) under dry condition, C_{12+} olefins either did not form (in the presence of montmorillonite) or formed initially in very low amounts (1.5% of total saturated hydrocarbons generated from kerogen heated alone at 300 °C for 2 hours), and almost disappeared completely with prolonged heating time (0.04%, 300 °C, 2 hours). Furthermore, no olefins were detected under dry condition in an experiment where the temperature was gradually increased by 1 °C per week over a 6-year period⁸. These results indicate that hydrous pyrolysis with excess water does not necessarily have an advantage over dry condition in this respect. Moreover, the low olefin quantities generated in

the presence of montmorillonite under dry conditions (Fig. 2) could indicate that smectite clays have a strong control in suppressing unsaturation in natural conditions.

Although rarely found in petroleum source rocks or in crude oils, olefins exist in small amounts in surface sediments where their source is probably biogenic^{17,18}. However, several reports have shown their presence in mature sediment or oils, where they formed by thermal cracking of organic molecules^{19–22}. They probably form continuously in small amounts, but due to their reactivity, they will disappear with geological time by acquiring hydrogen from other organic molecules, minerals or hydrogen gas. This suggests that olefins are initially produced in small quantities, under either hydrous or dry conditions and represent a transitory unstable intermediate in the process of generation of liquid or gaseous hydrocarbons during the thermal maturation of kerogen.

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Stable isotope evidence for chemosynthesis in an abyssal seep community

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Communities of abundant organisms, similar to those which surround the hydrothermal vents of the East Pacific Rise (EPR), occur in 3,266 m of water in the Gulf of Mexico. They were discovered with the *Alvin* at 26° 02' N, 84° 55' W in an area where pore waters seep from the Cretaceous limestones of the adjacent platform at the contact between the Florida Escarpment and the Holocene hemipelagic sediments of the Abyssal Gulf. Because of the unusual abundance of organisms in this deep-sea environment, and the taxonomic similarities to the chemosynthetically based communities which surround the hydrothermal vents on the EPR, the seep communities are suspected to exist on a local non-photosynthetic food source¹. We have now measured isotope ratios

in tissues of organisms which surround these saline seeps to determine the origin of the local food chain. The tissues have extremely negative $\delta^{13}\text{C}$ (-42 to -77% PDB) and $\delta^{15}\text{N}$ (-2.72 to -9.34% air) values. Such highly fractionated carbon and nitrogen isotopes are unknown in food chains based on photosynthesis, suggesting that these communities are chemosynthetic. The cause of this extreme fractionation is attributed either to assimilation from local supplies of isotopically depleted methane and ammonium or to biochemical fractionation. The tissues of these animals contain significant amounts of ^{14}C ($\sim 60\%$ modern), so the source of this fractionated carbon is not predominantly fossil methane.

The seep communities occur on patches of irregularly distributed black sediments, 5–10 m in diameter, which are found directly at the base of the Florida Escarpment. These black sediments contain up to 38% iron sulphide minerals¹, and up to 8% organic carbon. These organic carbon concentrations are unusually high for young abyssal sediments and are clearly higher than the concentrations in the surrounding tan-coloured hemipelagic sediments. The pore fluids in the upper few centimetres of the black sediments contain chloride ions at up to 25.61 g per kg, which is a 32% enrichment with respect to ambient sea water. Such steep concentration gradients can only persist where advection of pore fluids is occurring. The seeping hypersaline fluids arrive at the sea floor at near ambient temperatures, and carry reduced inorganic compounds (such as H_2S and NH_4)¹ which are potential energy suppliers for chemosynthesis².

The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of mytilid mussel, vestimentiferan, and trochid gastropod tissues from the seep sites are much lower than occur in photosynthetic food chains or within the dissolved chemical species in sea water (Table 1). Seep mussel tissues contain the lightest $\delta^{13}\text{C}$ (-74.3% PDB) yet reported from a contemporary organism^{3–5}, and $\delta^{15}\text{N}$ is as low as -9.34% (atm). The $\delta^{13}\text{C}$ values of extractable organic carbon in the black sediments average $-80.4 \pm 4\%$ PDB.

Characteristic ranges of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are known for most of the carbon and nitrogen sources available to the seep organisms (Fig. 1). Normally the $\delta^{13}\text{C}$ in animal tissue is approximately equal to that of its food^{3–7}. Neither nitrogen fixation nor nitrogen assimilation from nitrate and ammonium are known to be associated with significant isotope fractionation^{8,10}. Consequently, the $\delta^{15}\text{N}$ ratios of primary producers reflect the nitrogen source. Heterotrophic processes in the food chain tend to increase the $\delta^{15}\text{N}$ values in animal tissues in successive trophic levels¹¹. Thus, the $\delta^{15}\text{N}$ values in animals are usually heavier than the assimilated nitrogen.

Table 1 ^{13}C and ^{15}N values for samples from seep environment

	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
Organic material		
Mytilid tissue	-74.3 ± 2.0 (10)	$-9.34, -8.57$
Trochid gastropod tissue	-59.9 ± 0.7 (2)	-3.62
Vestimentiferan tissue	-42.7 ± 0.7 (3)	$-2.80, -2.72$
Extracted organic carbon from sediment	-80.4 ± 0.4 (3)	
Carbonate material		
Mytilid shell	-4.8 ± 0.9 (12)	
Trochid gastropod shell	-3.1 ± 0.4 (2)	
Escarpment limestone	3.2 ± 0.05 (2)	

Mean values ($\pm$ s.d.) for $\delta^{13}\text{C}$ (PDB) (with the number of measurements in parentheses) and individual values for $\delta^{15}\text{N}$ (air) of carbon and nitrogen-containing components of the seep environment are given. $\delta = 1,000 (R_{\text{sample}} - R_{\text{standard}})/R_{\text{sample}}$, where R is the ratio of two isotopes. The CO_2 was evolved from carbonates by reaction with phosphoric acid at 50°C and from the organic carbon by oxidation in a vacuum with cupric oxide at 850°C for 6 h, before measurements were made with a Micromass 602 mass spectrometer at SIO. The corporation Global Geochemistry measured the $\delta^{13}\text{C}$ on one mytilid tissue sample (-73.76% PDB), the $\delta^{13}\text{C}$ of the extractable organic carbon from the black sediment, and all the $\delta^{15}\text{N}$ values. The inter- and intra-sample $\delta^{13}\text{C}$ variability are similar for the mytilid tissues.

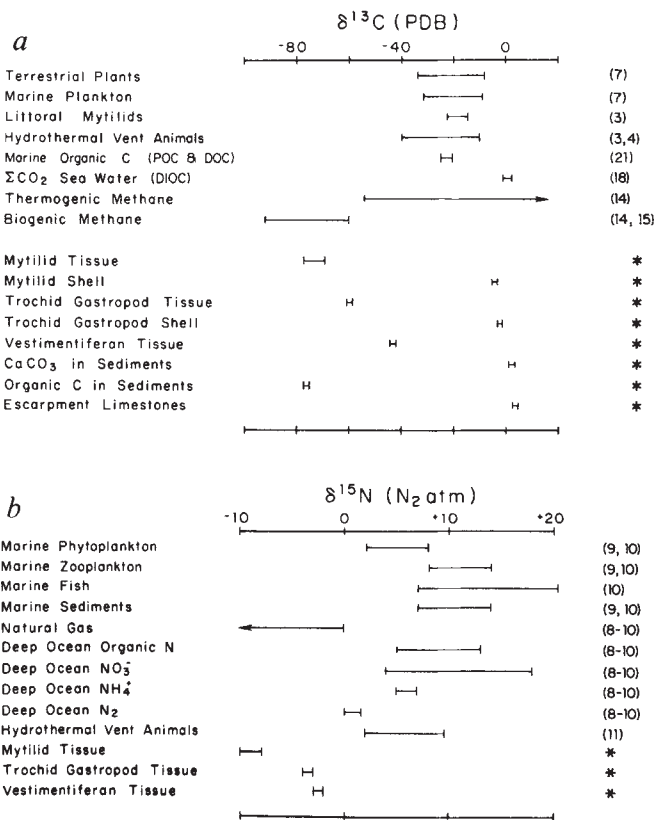


Fig. 1 Ranges of $\delta^{13}\text{C}$ (a) and $\delta^{15}\text{N}$ (b) in components of the seep environment and in possible sources of these components, with references to their sources. The asterisks indicate values reported in the present paper.

The values of carbon and nitrogen isotopes measured on organic material within the seep environment are outside the range found in tissues derived from photosynthetic food chains. This suggests that the organic matter which forms the base of the seep food chain did not settle from surface waters, but is generated through local primary production.

The mussels and vestimentiferans are thought to exist with endosymbiotic bacteria which are primary producers^{12,13}. Although both groups of macrofauna contain extremely light carbon, they differ in their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Table 1 and Fig. 1). This suggests that either the sources of the assimilated elements or the biochemical pathways and resulting fractionation patterns differ between these organisms.

The seep vestimentiferans have $\delta^{13}\text{C}$ values similar to those of animals in the chemosynthetic communities which surround the East Pacific Rise hydrothermal vents (Fig. 1). In the hydrothermal vent communities of the East Pacific Rise, the oxidation of sulphides is the dominant energy source and the tissue carbon appears to be derived primarily from dissolved inorganic carbon (DIOC) in sea water^{2,4}.

The unusually depleted $\delta^{13}\text{C}$ values of the mytilid tissues and the disseminated organic carbon in the seep sediments suggest primary production by methylotrophic bacteria, which assimilate highly fractionated carbon in the form of methane. The concentration of methane at the seeps was not measured. Thermogenic methane $\delta^{13}\text{C}$ values are more positive than -55% PDB¹⁴, and would require additional fractionation. However, biogenic methane concentrations contain isotopically light $\delta^{13}\text{C}$ (-60 to -90% PDB) and are known to rise in anoxic systems after all existing sulphate has been reduced to sulphide^{14,15}. The presence of hydrogen sulphide and ammonium in the seeping fluids attests to the reducing conditions within the platform¹, and is consistent with the existence of biogenic methane.

$^{14}\text{C}/^{13}\text{C}$ ratios were measured in the shell and tissue of seep mussels and in the tissue of the seep vestimentiferans with the

Table 2 Carbon-14 concentrations measured in the shell and tissue of seep mussels and in the tissue of seep vestimentifera

Mytilid mussel Sample no.	Shell	Tissues
2	84.8 ± 2.3%	43.3 ± 1.5%
3	76.2 ± 1.7%	75.3 ± 1.7%
4	84.2 ± 1.3%	69.6 ± 0.9%
Vestimentiferan		
A		58.1 ± 1.3%
B		57.6 ± 0.8%

The University of Arizona's tandem accelerator mass spectrometer^{16,17} was used. Values are $\delta^{13}\text{C}$ corrected using the mean values for the same samples as given in Table 1. Values are % modern carbon.

University of Arizona's tandem accelerator mass spectrometer^{16,17} (Table 2). The seep mussel shells contain on average 81.7% modern carbon, which is close to the expected ^{14}C content of dissolved CO_2 in the deep-water reservoir¹⁸. Although the ^{14}C content of mytilid tissues is quite variable, the mytilid and vestimentiferan tissues average, respectively, 19.0% and 23.8% less modern carbon than the mytilid shells.

Methane originating in the interior of the adjacent platform should not contain detectable ^{14}C because of its long separation from sources of atmospheric carbon. If methane from the adjacent platform is assimilated by these organisms, their tissues should be diluted by ^{14}C -free carbon relative to their shells which should have the ^{14}C content of DIOC. The $\delta^{13}\text{C}$ values in the carbonate of the mussels and the gastropod shells (Table 1) are not significantly different from those typical of abyssal DIOC which is the usual source of molluscan carbonate^{18,19}. Moreover, the carbonates in the adjacent limestone escarpment have $\delta^{13}\text{C}$ values typical of Cretaceous limestones. Apparently, the isotope values of the DIOC present at the seeps are not unusual.

The ^{14}C concentrations (~60% modern) in tissues of the seep organisms restrict fossil methane sources to being less than a quarter of their nutritional carbon. Assimilation of this limited amount of isotopically depleted fossil methane does not explain the observed $\delta^{13}\text{C}$ values.

The following model is proposed to explain both the ^{14}C and the $\delta^{13}\text{C}$ values. First, ambient DIOC is fixed as the tissue carbon of chemosynthetic bacteria which utilize either the sulphide or ammonium in the seeping pore fluids as an energy source. Some of this organic carbon is incorporated in the sediments which are bathed in the advecting pore fluids. In the reducing environment of the shallow subsurface, methanogenic bacteria may actively reduce organic matter to biogenic methane. The resulting methane would contain ^{14}C concentrations approximately equal to those of sea water DIOC, $\delta^{13}\text{C}$ values of -60 to -90‰ PDB, and would be available for methylotrophic assimilation²⁰. This scenario requires efficient recycling of organic carbon within the seep environment, active methanogenesis in the organically rich reduced black sediments, and a succession in the types of reduced compounds being used as energy sources by chemosynthetic bacteria at the seeps.

Carbon and nitrogen isotopes in organic material from the Florida Escarpment seep communities indicate that chemosynthesis is occurring. Although the $\delta^{13}\text{C}$ values suggest that the carbon source might be methane, mussel tissues contain only 19% less ^{14}C than their shells, suggesting that the largest carbon source is not ^{14}C -free methane coming from the adjacent continental margin platform, but ultimately DIOC from sea water. Methanogenesis in the seep sediments could provide fractionated carbon for methylotrophic organisms, but would require a complicated pattern of succession within these communities. The potential of biochemical fractionation of carbon during chemosynthetic primary production processes has not been adequately investigated, but is also a potential cause of the extreme isotope depletion.

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Development of widespread mangrove swamps in mid-Holocene times in northern Australia

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Mangrove forests in northern Australia typically occur as fringes along tidal estuaries and relatively sheltered coasts. Radiocarbon dating evidence from the South Alligator River, presented here, suggests that extensive mangrove swamps developed between 6,500 and 7,000 yr ago and flourished for about 1,000 yr. Pollen analysis of a stratigraphic core at a mid-plains site links the growth of these forests with the interaction of sea-level change and sedimentation. This was succeeded by the development of flood-plains with tidal river channels, a dramatic ecological change that has implications for all coastal and nearshore systems.

Northern Australia is drained by several large rivers which flood annually in the wet season and which are tidal for a substantial length under the influence of high (macrotidal) tidal ranges. Mangrove communities in these tidal rivers are typically narrow fringes, often discontinuous, confined to channel banks and the edges of tidal creeks. Closed mangrove forests are virtually restricted to sites of active sedimentation such as prograding sections of coast, shoaling mid-channel islands, and channel point bars. Bare high-tidal and supratidal flats, or grass and sedge-covered plains, now extend across the estuarine or deltaic surfaces of Holocene age in these areas. From Broome in the west to Arnhem Land in the east, the tidal rivers drain a weathered lateritic hinterland or dissected Precambrian plateau. Maximum spring tidal range varies from 12 m at Derby to 6 m at the north Arnhem Land coast. Tidal influence extends more than 100 km upstream along some rivers. Monsoonal rainfall in the December-March wet season ranges up to ~1,200 mm in the Darwin-Arnhem Land region, and is 600-1,000 mm in north-

western Australia (Fig. 1). High runoff efficiency late in the wet season leads to extensive freshwater flooding of estuarine and deltaic plains adjacent to the tidal rivers. In the wetter part of the region (Fig. 1), these plains (known in Australia as blacksoil plains) are largely covered by sedges and grasses¹. There are vast areas of bare high-tidal and supratidal mudflats in the lower-rainfall areas².

In recent years, several projects have investigated the morphostratigraphy of estuarine and coastal plains of northern Australia. Shallow coring and riverbank exposures reveal an organic-rich facies at shallow depth in King Sound^{3,4}, the Fitzroy River⁵, the Ord River², and in the Daly and Alligator rivers⁶ (for localities see Fig. 1). A similar facies underlies the prograded coastal plain of Princess Charlotte Bay, eastern Cape York Peninsula⁷, but seems to be absent beneath the much larger prograded coastal plains of the southern Gulf of Carpentaria⁸. This organic facies has been shown by pollen analysis to represent former mangrove forests at Princess Charlotte Bay⁷. Similar pollen analysis during our present study demonstrates the mangrove origin of this facies in the South Alligator River. River bank exposures and drill recovery of the facies include mangrove roots, and sub-fossil mangrove molluscs, lobsters and crabs. These are observed at many other sites as well as at ours, confirming the mangrove-forest origin of the facies^{3,5}.

Radiocarbon dating of this mangrove facies has been done at several sites in northern Australia. Where it occurs at a shallow depth (2–7 m) beneath the floodplains and tidal flats, the facies is generally shown to have formed between 5,500 and 7,500 radiocarbon yr BP, for example, in the Fitzroy (5,800–7,500 yr BP)⁵ and the Ord (6,700 yr BP)². Our own dating of this facies in the South Alligator has been much more extensive than these earlier studies. Figure 2 indicates the location and ages of 33 dated mangrove wood samples from beneath the South Alligator plains. Table 1 lists relevant sample data. The 33 results are scattered throughout the plains and show no geographical trends of age values. Ages range from 5,370 to 6,860 yr BP. Many of the results in Fig. 3 represent paired samples, where one member of the pair is from near the top of the mangrove facies (usually within 3 m of the ground surface), and the other is from near its base (usually 5–7 m below ground surface). In some cases, there is no statistical age difference between the upper and lower samples; in others the lower sample is older by 500–1,000 yr. The results show that extensive mangrove forest was established throughout the South Alligator plains region around 6,500–7,000 yr ago, and persisted until ~5,500 yr ago. It seems likely that mangrove flourished throughout most of the area during this time.

We link the development of this big mangrove swamp to effects of relative sea-level change. In Australia, rising sea level reached its present position 6,500 ± 250 radiocarbon yr ago⁹. In the north-east, sea level reached its maximum, about 1 m higher than present relative to the mainland coast, around 6,000 yr ago; since then it has fallen very slowly and generally uniformly¹⁰. Radiocarbon in Table 1 and Fig. 2 show that the large mangrove swamp in the South Alligator flourished at the time when the sea level was stabilizing. Before 7,000 yr BP, the broad valley of the South Alligator River was infilling with sediment while the sea level rose. Stratigraphical drilling shows that most of this sediment accumulated in a shallow estuarine environment. As sea level reached its present position, sites for mangrove colonization developed throughout the shoaling estuary. Continued sedimentation, after the sea level stabilized, progressively eliminated intertidal environments, effectively choking out most of the mangrove swamp by ~5,500 yr BP. Vertical accretion led to the formation of bare high-tidal flats in the western (lower rainfall) area², and vegetated plains in the most northern region.

This relationship between changing sea level, sedimentation and mangrove growth and decline is demonstrated by pollen analysis of a stratigraphical core at a mid-plains site (core SA40, location shown in Fig. 2). Essential features of the core are shown in Fig. 3. The core exhibits the following sequence of zones: zone 1 (>8 m depth), estuarine muds and finely sandy

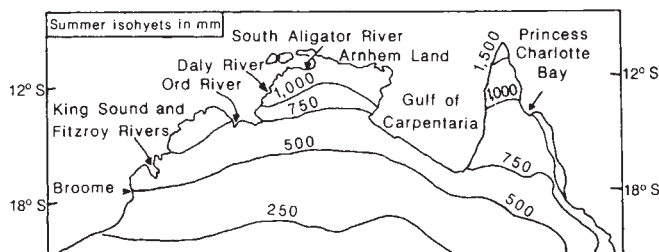


Fig. 1 Localities mentioned in the text, including mid-Holocene extensive mangroves. Isohyets show wet season (December–March) rainfall (atlas of Australian Resources 1973).

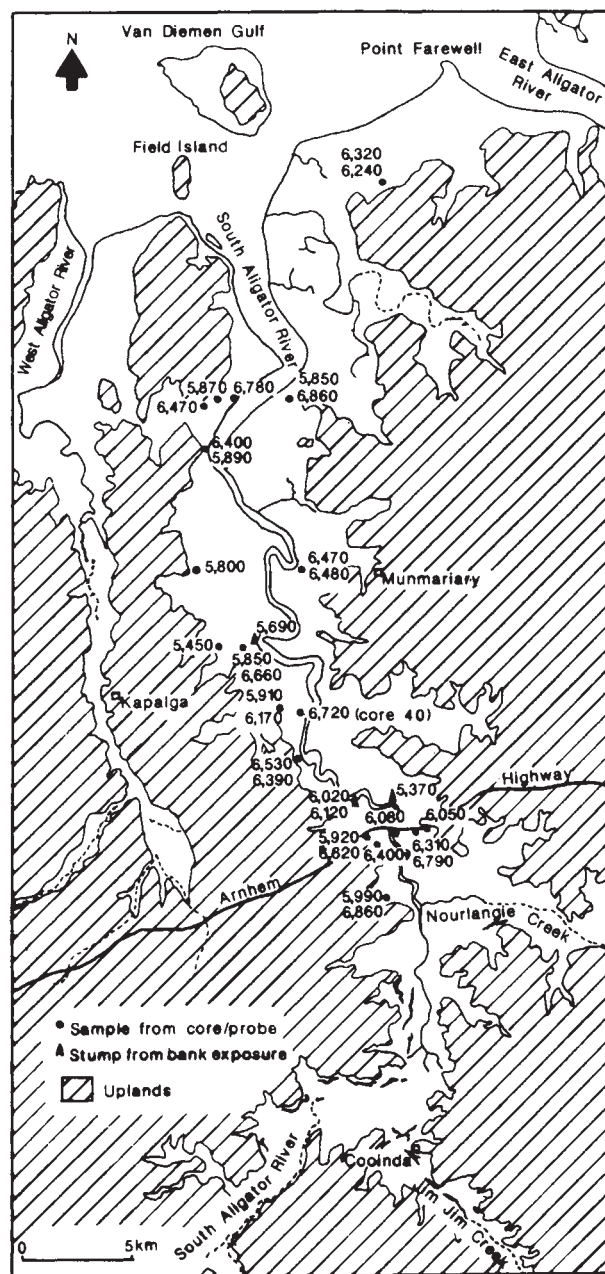


Fig. 2 South Alligator River and floodplain (Northern Territory) showing locations and ages of 33 drill holes through buried mangrove facies. Paired age results are from near the top and bottom of the regressive mangrove horizon.

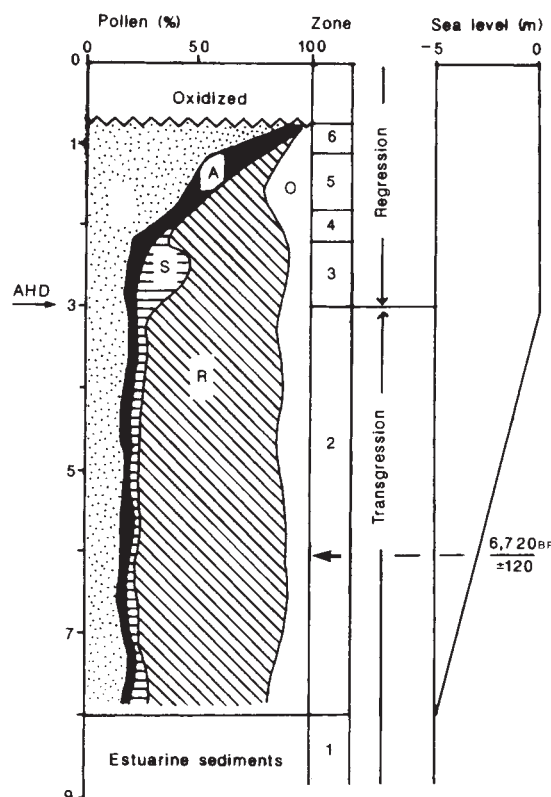


Fig. 3 Pollen diagram of South Alligator floodplain drillcore 40 (for location, see Fig. 2). The pollen zones are discussed in the text. Basic patterns of sea-level rise and post-6,000 yr BP stability are shown on the right. P, Plains vegetation (cyperaceae + Poaceae); A, *Avicennia*; R, *Rhizophoraceae*; S, *Sonneratia*; O, other (dominantly *Myrtaceae* + herbs).

Table 1 South Alligator radiocarbon sample information

Field code	ANU lab. no.	Grid ref. (1:100,000 sheet)	Depth (m)	Material used for dating	Age and error (Myr)
SAH 28/1*	3929	KG 286523	2.45	MW	6,320 ± 170
SAH 28/2	3930	KG 286523	3.95	MW	6,240 ± 130
SAH 56/6	4010	KG 158338	8.10	FMF	6,780 ± 90
SAH 57/1	3923	KG 149337	2.50	MW	5,870 ± 220
SAH 32/901	4115	KG 215350	3.95	FMF	5,850 ± 280
SAH 32/902	4008	KG 215350	9.15	FMF	6,860 ± 150
SAH 58/2	3924	KG 140337	3.70	MW	6,470 ± 110
SAH 7/310	3782	KG 140305	1.80	FMF	6,400 ± 820
SAH 7/323	3780	KG 140305	3.40	FMF	5,890 ± 230
SAH 46/4	3866	KG 136213	3.75	FMF	5,800 ± 120
SAH 52/2	4003	KG 220203	2.40	FMF	6,470 ± 130
SAH 52/3	4009	KG 220203	3.65	MW	6,480 ± 110
SAH 60A/1	4006	KG 178142	MLW	MW	5,690 ± 90
SAH 60/2	4004	KG 173142	4.60	FMF	6,660 ± 100
SAH 60/4	4005	KG 173142	7.30	FMF	5,850 ± 110
SAH 62/2	3989	KG 155139	4.60	FMF	5,459 ± 230
SAH 13/516	4116	KG 199089	4.70	VFMF	5,910 ± 190
SAH 13/528	3924	KG 199089	7.20	MW	6,170 ± 200
SAH 40/1	3863	KG 213086	5.60	FMF	6,720 ± 120
SAH 1/30	3778	KG 214047	4.70	FMF	6,390 ± 130
SAH/32	3783	KG 214047	4.80	FMF	6,530 ± 690
SA 80/2	3937	KG 282003	MLW	FMW	6,020 ± 90
SA 80/1	3936	KG 292003	MLW	MW	6,120 ± 90
SA 80/14	4259	KG 290009	MLW	MW	5,370 ± 80
SAH 75/4	4128	KG 292987	4.60	FMF	6,080 ± 170
SAH 65/4	4052	KG 313987	2.45	FMF	6,050 ± 130
SAH 66/4	4051	KG 306986	5.50	FMF	6,310 ± 110
SAH 66/6	4055	KG 306986	7.90	FMF	6,790 ± 140
SAH 73/3	4130	KG 270979	1.50	FMF	5,920 ± 100
SAH 73/5	4129	KG 270979	3.00	FMF	6,620 ± 150
SAH 69/1	4122	KG 274970	2.10	MW	6,400 ± 240
SAH 67/6	4050	KG 288917	4.30	FMF	5,990 ± 110
SAH 67/10	4049	KG 288917	6.70	FMF	6,860 ± 110

SAH, South Alligator Hole; MLW, mean low water; MW, mangrove wood; FMF, fine mangrove fragments; VFMF, very fine mangrove fragments; FMW, fossilized mangrove wood.

muds, partly laminated; zone 2 (3.0–8.0 m), mangrove facies dominated by species of *Rhizophoraceae*; zone 3 (2.4–3.0 m), mangrove facies dominated by *Rhizophoraceae* with an admixture of *Sonneratia*; zone 4 (1.8–2.4 m) returns to mangrove facies dominated by *Rhizophoraceae* with other accessory mangroves; zone 5 (1.0–1.8 m) is a more sparse mangrove facies dominated by *Avicennia*; this passes into zone 6 (<1.0 m) where grasses and sedges dominate. We refer to zones 1 and 2 as the transgressive sequence, believing that it accumulated during the last stages of sea-level rise, and to zones 3–6 as the regressive sequence which accumulated after the sea level stabilized.

Mangrove deposition in zone 2 of Core SA40 is believed to have commenced when the sea level was rising, as its base is 5 m below AHD (Australian Height Datum, which approximates to mean sea level) (Fig. 3), or about 5 m below the general lower limit of vigorous mangrove growth (based on our levelled surveys of mangrove forests near the present South Alligator River mouth). A point 3 m below AHD in zone 2 is dated as $6,720 \pm 120$ yr BP, which is consistent with this interpretation (Fig. 3). The regressive sequence above 3.0 m depth shows succession from *Sonneratia* through *Rhizophoraceae* then *Avicennia* to sedge/grass communities. This vertical succession closely resembles the spatial zonation from lower intertidal mangrove through to plains vegetation, typical of present-day coastal and lower estuarine mangrove swamps. Hence, the sequence demonstrates establishment of mangrove swamp during the last stage of the sea-level rise, followed by choking-out of the swamp by continued sedimentation, leading to establishment of blacksoil plains.

We conclude that the extensive mangrove swamps which developed from 6,500 to 7,000 radiocarbon yr ago and flourished for ~1,000 yr, were succeeded by developing floodplains with tidal river channels and relatively little mangrove by ~5,500 yr BP. This dramatic ecological change was a response to sedimentary infill adjusting to the change from rising to stable sea level. A similar pattern has apparently occurred in other tidal rivers of northern Australia, with each river differing according to its own particular sediment output and the dimensions of its lower valley and estuarine system. In this light, no change of hydrology is invoked to explain the past existence of extensive estuarine swamps. This is contrary to Jennings⁵ who interpreted the Fitzroy mangrove swamp phase as the result of a wetter climate. The rise and decline of this short-lived phase of widespread mangrove swamps will have had ecological effects throughout coastal and nearshore systems. The South Alligator mangrove alone was about 80,000 hectares in area, ~8–10% of the present total mangrove area of tropical Australia. It will be interesting to discover whether any changes of mangrove-based food chains can be detected in related fossil or even in archaeological deposits.

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Note added in proof: We are advised by the ANU Radiocarbon Laboratory that some of these results may be altered slightly, within one standard error, by future reevaluation of counting backgrounds.

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Potentially toxic concentrations of triethyl lead in Black Forest rainwater samples

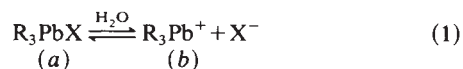
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Recently, speculation has grown that the European forest damage may be caused by the continual exposure of trees to rainwater containing trialkyl lead salts¹ (R_3PbX ; R = ethyl, methyl). These are degradation products of tetraalkyl lead (R_4Pb) which are added to petroleum as anti-knock agents; they inhibit tubulin polymerization^{2,3} and are highly toxic to mammalian and plant cells⁴. Here we describe the analysis of rainwater samples collected at two sites in the Black Forest, FRG. Total lead content was measured by atomic absorption and found to be in the range previously determined for rural areas⁵. The portion of R_3PbX was assayed by the inhibition of pork brain tubulin polymerization. R_3PbX was present in one-third of all rainwater samples. The highest concentration was $0.3 \mu M$, that is 10^3 times higher than previously reported⁵. For comparison, $1 \mu M$ R_3PbX killed soybean cells or neuroblastoma cells in culture⁴.

Airborne organolead has been analysed by various methods^{6,7}. Average values reported from several laboratories suggest that alkyl lead concentrations of $\sim 100 \text{ ng m}^{-3}$ exist in urban air, and $10\text{--}20 \text{ ng m}^{-3}$ in residential or rural areas, mostly as R_4Pb . In garages or in the environment of petrol stations, quantities may increase to $2 \mu \text{g m}^{-3}$. None of the methods used allowed the detection of the less volatile degradation products of the anti-knock agents, such as trialkyl lead or dialkyl lead salts (R_3PbX or R_2PbX_2 , respectively). There are, however, indications that non-negligible amounts of these compounds exist in air⁸ either in gaseous phase, or associated with particulate matter^{9,10}.

In the absence of water, trialkyl lead compounds have four covalent ligands (reaction 1a); the presence of water leads to the formation of salts (reaction 1b) with the driving force of this process being solvation, particularly of the anion¹¹.



Because of this feature, one would expect to find R_3PbX in rain and fog rather than in air. However, attempts to determine

R_3PbX in samples of rain or surface water had been unsuccessful¹² until de Jonghe and co-workers described a specific extraction procedure¹³. By using this method, some rainwater samples collected in the city and in the rural environment of Antwerp were analysed for R_3PbX (ref. 5). The concentrations found were as low as 38 and 28 ng l^{-1} , respectively, accounting for $<0.1\%$ of total lead. Such low concentrations were regarded as non-hazardous for the environment.

The present study shows that the situation at places located in the middle of the European continent, for example on the western rim of the Black Forest, may differ from the coastal region. About 50 samples of rainwater were collected between September and December 1984 at the meteorological station on top of the Feldberg, near Freiburg ($1,400 \text{ m}$ above sea level (a.s.l.)), and in the environment of Loffenau, in the northern part of the hill country close to Baden-Baden (600 m a.s.l.). Reports have come from both locations of damage to fir (*Abies alba* L.) and spruce (*Picea abies* L.). For the analyses care was taken that all local sources of organolead were excluded in that the sampling sites were located several kilometres away from roads or from parking areas.

Lead was present in $\sim 80\%$ of all samples, in concentrations between 0.01 and $1 \mu M$ (Fig. 1), which is in good agreement with the value ($0.15 \mu M$) reported previously⁵. For evaluation of R_3PbX , we established a biological assay, using the recent finding^{2,3} that trialkylated lead compounds can inhibit microtubule assembly. Because inorganic lead salts do not interfere (only at concentrations of $>100 \mu M$ did lead acetate cause precipitation of the protein; C.S., unpublished data), separation of inorganic lead was not necessary. Full inhibition was obtained at $7.5 \mu M$ triethyl lead chloride (Et_3PbCl), as monitored by spectrophotometry at 350 nm . The inhibitory capacities of the rainwater samples were expressed as μM Et_3PbCl , which was used for calibration. Typical curves are shown in Fig. 2.

About one-third of the rainwater samples contained R_3PbX in concentrations between 0.01 and $0.3 \mu M$ (solid bars in Fig. 1). These concentrations are up to 2,000 times higher than that ($0.00014 \mu M$) previously reported⁵. Considering that in some samples R_3PbX accounted for $>50\%$ of total lead, it seems reasonable to assume that the inorganic lead (PbX_2) found in the rainwater had also been transported there as organolead. We assume that uncombusted anti-knock agent (R_4Pb) escaping continuously, for example, from idling engines or petrol stations, is distributed in the atmosphere. While being transported over long distances, it may be degraded to R_3PbX . Because of its solubility in water, R_3PbX probably enters into water droplets of fog or clouds and finally accumulates in precipitation. Further

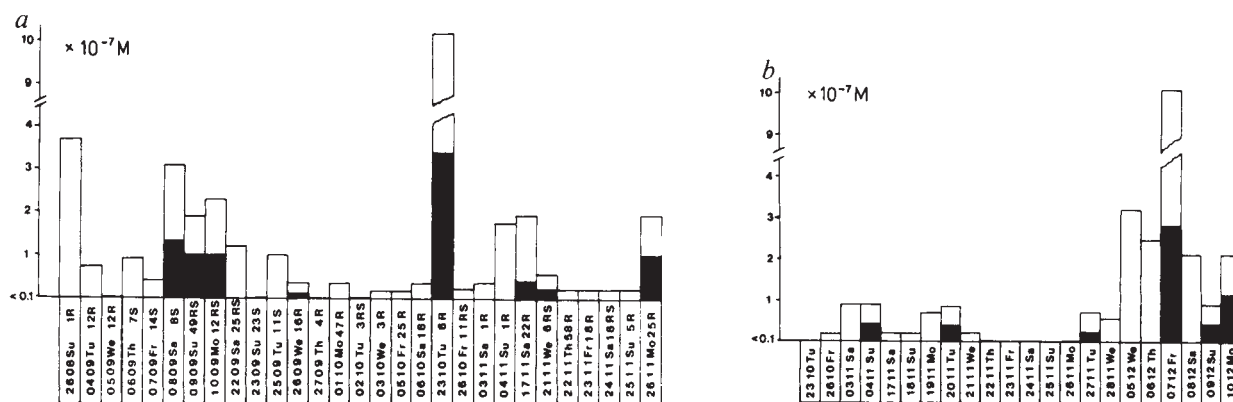


Fig. 1 Concentrations of total lead (open bars) and trialkyl lead (solid bars) in rainwater samples from: *a*, the meteorological station on top of the Feldberg (Black Forest, FRG); *b*, the Forest of Loffenau (20 km west of Baden-Baden, Black Forest, FRG). Precipitation was collected in glass vials placed inside a closed black polyethylene box ($0.7 \times 0.7 \times 0.7 \text{ m}$). The sampling method did not exclude the possibility that in periods between precipitations a dry deposition of lead had taken place. The samples were filtered, if necessary, to remove dust particles. Filtration did not significantly alter the total lead content. Values below the bars represent the date, day, volume (in $\text{mm m}^{-2} \text{ day}^{-1}$) and type (R, rain; S, showers) of precipitation. Lead was measured by atomic absorption using a Perkin Elmer 4000 equipped with a HGA-400 graphite cuvette. $20\text{-}\mu\text{l}$ samples of rain, 0.2% in HNO_3 , were dried at 100°C for 30s, carbonized at 500°C for 20s and atomized at $2,300^\circ\text{C}$ for 10s. $\text{Pb}(\text{NO}_3)_2$ -standard (Merck, Darmstadt) was used for calibration. Concentration of trialkyl lead was determined by inhibition of microtubule assembly as described in Fig. 2 legend.

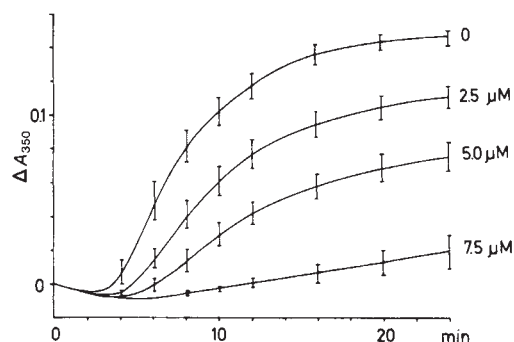


Fig. 2 Biological assay for determining trialkyl lead concentrations in evaporated rainwater samples. Pork brain tubulin, prepared as in ref. 2 but not separated from microtubule-associated proteins (1-ml samples, 15 μ M, in a quartz cuvette, in buffer containing 0.1 M PIPES, 1 mM MgCl_2 , 0.1 mM EDTA and 1 mM EGTA, pH 6.9), were polymerized by adding GTP to a final concentration of 1 mM and warming the samples from 0 to 37 $^{\circ}\text{C}$. Polymerization was monitored by absorbance at 350 nm on an Aminco DW 2 spectrophotometer. Aliquots of evaporated rainwater samples (10–50 μ l) were added to the tubulin immediately before polymerization began. For calibration, Et_3PbCl (Ventron, Karlsruhe; purified by column chromatography on silica gel with benzene containing 5% acetic acid) was used. During evaporation of the rainwater samples ($\sim 1:200$), total lead content decreased due to the adsorption of R_3PbX to the glass surface, probably as reaction (1a) formed in anhydrous conditions. Repeated washings of the flasks recovered trialkyl lead with 90% yield. The evaporated samples were assayed in at least two dilutions corresponding to concentrations between 2.5 and 7.5 μ M Et_3PbCl . Each of the solid bars in Fig. 1 represents a value interpolated from at least two of the inhibition curves shown here.

degradation of R_3PbX to PbX_2 certainly occurs, but must be slower than generation of R_3PbX from R_4Pb .

Most of the Feldberg samples containing R_3PbX in concentrations $\geq 1 \times 10^{-7}$ M were collected in typical weather conditions, that is, with wind from the south-west. However, the number of samples was limited, and wind from the south-west prevails on most days (70%) with precipitation. Therefore, the answer to the question of whether wind from this direction is concomitant with high organolead pollution in rain, will require a longer period of observation.

The analytical procedure recently described¹³ does not differentiate between the various possible species of R_3PbX such as Et_3PbX , Et_2MePbX , EtMe_2PbX and Me_3PbX . These compounds, however, differ in their cytotoxic activity, as can be shown in experiments with algae¹⁴, in which Et_3PbX is considerably more toxic than Me_3PbX . Accordingly, the measurement of organolead concentration will provide reliable information on cytotoxicity only in cases where the composition of the mixture is known. On the other hand, considerable evidence has accumulated suggesting that antimicrotubular activity is closely related, or even identical, to cytotoxicity. Such a correlation has been shown for mammalian cells^{2,4} as well as for plant cells such as algae¹⁴. Similarly, the decrease of the mitotic index, as observed in the tissue of onion^{15,16} (*Allium cepa*), is most easily explained by assuming a disturbance of the microtubular system. Therefore, measuring the antimicrotubular activity is thought to give the most reliable information on the ecological menace residing in a lead-polluted rain sample.

The highest concentrations of R_3PbX determined in rainwater were only three times lower than the concentration of Et_3PbCl that is toxic for neuroblastoma or soybean cells. In this context, it is interesting that needles of coniferous trees, such as fir and spruce, accumulate Et_3PbCl several-fold from dilute aqueous solutions¹. For example, 1 g of fresh plant tissue incorporated 1.6 μ g of Et_3PbCl from a 0.3 μ M solution within minutes. Provided the toxin is freely distributed between the extracellular and intracellular space, the concentration of Et_3PbCl in the

aqueous compartments of the plant tissue may rise to $>1 \mu\text{M}$, that is, to potentially phytotoxic concentrations.

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Archosaur ankles and the relationships of the thecodontian and dinosaurian reptiles

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Several attempts have been made to outline the early evolution and radiation of the archosaurs through a study of the morphology and function of their ankle joints^{1–6} (Figs 1a, 2). Here we present new interpretations of the evidence which show that several aspects of archosaur ankle anatomy have been misunderstood, and we re-state the case for the 'advanced mesotarsal' (AM) ankle of the dinosaurs being derived from an intermediate stage and not directly from the 'primitive mesotarsal' ankle of a proterosuchid thecodontian (Fig. 1b). Two modifications to the original scheme² are required. First, that *Euparkeria*-like ankles are basically 'proterosuchian', digitigrade and unlikely to be ancestral to any other form; the term 'modified primitive mesotarsal' is proposed to describe this pattern of ankle. The second modification is to recognize that no 'advanced mesotarsal (reversed)' ankle² has been described to date, and that previous allocations of forms to this type have been in error. Therefore, all forms with the AM ankle (Fig. 2) probably share a common ancestry. The 'advanced mesotarsal (normal)' ankle seems to be present in all known dinosaurs. The 'crocodile reversed' ankle is at present known only in the Ornithosuchidae². This supports earlier suggestions that the dinosaurs are a monophyletic group^{7–9}, a view not generally accepted⁵. The character state used here to define the dinosaurs is acquisition of the 'fully improved' gait, exemplified by the AM ankle¹⁰.

At the earliest level of organization in the archosaurs, in the thecodontian Proterosuchidae, the astragalus and calcaneum met in two pairs of articulation facets separated by a perforating foramen. The ankle hinge was mesotarsal, and the term primitive mesotarsal was used to describe this condition (Fig. 2, PM). Cruickshank² proposed that one or other of the complementary facets had later been elaborated into distinct but analogous rotating joints, and used the terms crocodile normal (CN) and crocodile reversed (CR) as suggested by Chatterjee¹¹ to describe these two conditions. In the CN state, a process on the astragalus

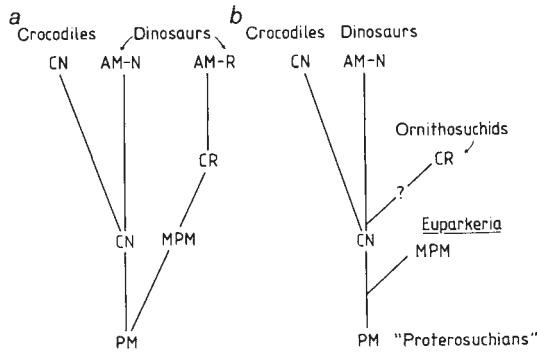


Fig. 1 *a*, Relationship of archosaurs by ankle pattern as suggested by Cruickshank². *b*, Revised relationships of archosaurs by ankle pattern as suggested in the present paper. PM, primitive mesotarsal; CN, crocodile normal; AM, advanced mesotarsal; AM-N, advanced mesotarsal (normal); AM-R, advanced mesotarsal (reversed); MPM, modified primitive mesotarsal; CR, crocodile reversed.

fits into a recess formed on the calcaneum, in front of and lateral to a 'tongue' which helps to stabilize the rotating joint (Fig. 2*a*, CN). The calcaneum grows a tuber calcis which points to the rear of the foot, a structure not seen in primitive archosaurs. This joint corresponds to the distal pair of facets joining astragalus and calcaneum in the earliest archosaurs (Fig. 2, CN). In the CR condition, the calcaneum develops a medial process which comes to lie in a hemicylindrical recess on the distal face of the astragalus. This joint corresponds to the proximal articulation facets of the primitive form. Once again a tuber calcis is formed (Fig. 2, CR). In both cases the other pairs of facets fail to make contact with each other and therefore the foramen is not enclosed; in each case the joint is intra-tarsal (cruro-tarsal).

There was a radiation of thecodontian lineages in the early to middle Triassic, the ankles of which were based on the proterosuchid PM ankle joint and which we call here modified primitive mesotarsal. This type of ankle is seen in the families Euparkeriidae, Erythrosuchidae and Proterochampsidae, none

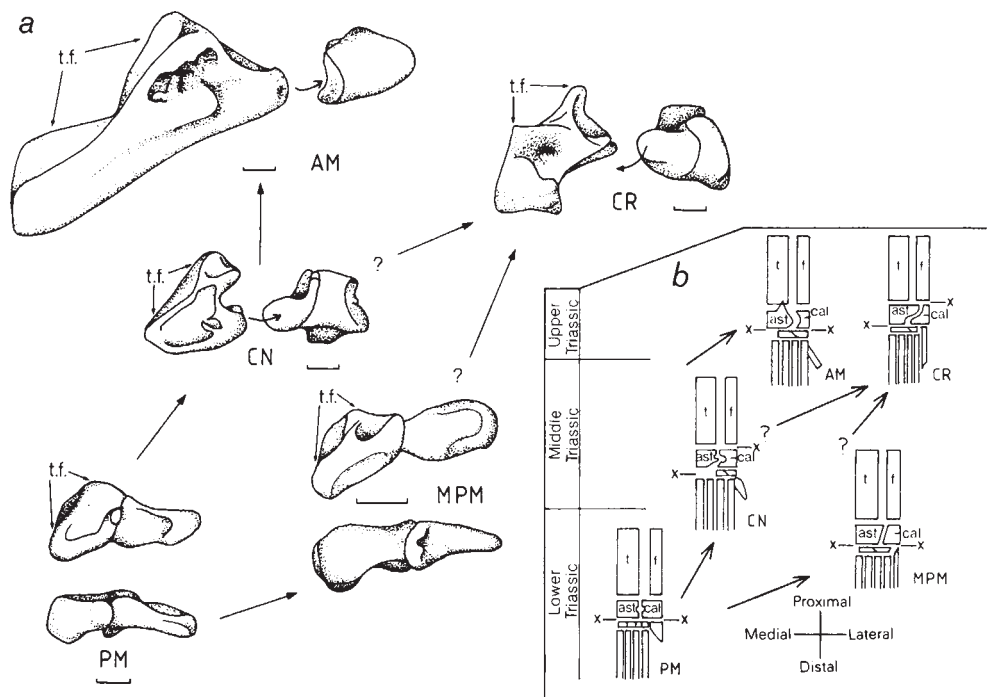
of which we believe had any descendants (Fig. 2, MPM). On the other hand, the major radiation that descended from the Proterosuchidae includes the crocodiles and all the dinosaurs, all of which possess a CN pattern of ankle (Fig. 2, CN). An analogous CR ankle is seen only in the Ornithosuchidae of the Upper Triassic (Fig. 2). Elaborating on the descriptions of these crocoid ankles, Cruickshank² suggested that several of the dinosaur lineages had CN ankles and postulated that others had CR ankles.

Three locomotory stages of the archosaurs⁶⁻¹² could be linked to the three morphological stages of ankle and foot. The earliest, present in the Proterosuchidae, was sprawling and PM in pattern. The intermediate condition was seen in the semi-erect CN and CR types, and the ultimate, fully erect (AM) type was seen only in the dinosaurs. In the original scheme² there were two AM ankles, AM-N (for CN-derived types) and AM-R (for the complementary forms)^{2,5}. The MPM type was believed then to fit in evolutionary terms between PM and CR ankles² (Fig. 1*a*).

The evolution of the AM-N ankle joint has been described in detail elsewhere^{2,13,14} and accepted by Chatterjee in a recent review⁵, thus confirming the apparent natural progression of ankle types from PM through CN to AM-N (Figs 1*b*, 2, AM). It is the status of the analogous AM-R ankle which is of importance here, as the resolution of this pattern of ankle joint has important implications for archosaur evolution in general and the dinosaurs in particular. If, as has been suggested^{2,5}, there are two patterns of AM archosaur ankle, the dinosaurs cannot be a monophyletic group, because the two patterns of ankle are not capable of changing, the one into the other, except in very special conditions^{2,4,5}.

Two taxa are particularly important in this study, *Euparkeria*^{2,15} and *Riojasuchus*^{2,16}. The anatomy of the ankle of *Euparkeria* is superficially what might be expected in an early example of a CR form. The calcaneum fits into a recess on the distal surface of the astragalus, and the perforating foramen is eliminated. However, the calcaneum retains the medial, posterior pyramid and the lateral tuber of the same pattern as in *Proterosuchus* and earlier forms². In this respect the illustration in Brinkman⁴ is at fault, for he gives the impression of a 'backwards' pointing tuber calcis, whereas it should be laterally directed (ref. 2, Figs 6-8; ref. 15, Fig. 13). In *Euparkeria*, with

Fig. 2 *a*, Astragalus and calcaneum from left ankles of: *Proterosuchus* (PM), anterior (upper) and distal (lower) views; *Neoaeosauroides* (CN), astragalus in anterior view, calcaneum rotated to show recess (arrowed) for reception of astragalar 'peg' (the medially pointing, spoon-shaped protuberance is the 'tongue', which stabilizes the calcaneum when rotating on the astragalus; see text); prosauropod gen. et sp. indet. (AM), detail as for CN; *Chanarsuchus* (MPM), detail as for PM; *Riojasuchus* (CR), calcaneum rotated to show 'peg'. *b*, Diagrammatic representations of the left limbs of the above taxa, as lettered, with timescale. All illustrations in *a* are redrawn from originals used in ref. 2 and have the tibial facets on the astragalus orientated in the same plane. Scale bar represents 1 cm in all cases. *Chanarsuchus* ankle (MPM) reproduced here for closer comparison with the others, as it is nearer their size than that of *Euparkeria*. ast, astragalus; cal, calcaneum; f, fibula; t, tibia; t.f., tibial facet; x-x, axes of ankle flexure; heavy arrows indicate lines of possible relationships.



a laterally directed tuber, any movement between astragalus and calcaneum would involve the calcaneum rotating on the axis of the posterior pyramid, in a proximo-distal direction; such movement would, however, be only minimal because of the stabilizing effects of the fibula proximally, and of the distal tarsals. Moreover, movement here is functionally unnecessary if it is assumed that *Euparkeria* was facultatively digitigrade¹⁵. Thus, *Euparkeria* has an ankle only slightly modified from the PM condition. The other forms with MPM ankles are *Erythrosuchus*, *Chanaresuchus* and their relatives¹⁷⁻¹⁹ which, along with *Euparkeria*, may represent an entirely separate radiation of early archosaurs (Figs 1b, MPM).

The CR ankle of *Riojasuchus* (and by implication, of the Ornithosuchidae in general) is structurally and functionally superficially similar to the CN type. However, the calcaneum is placed in a horizontal, hemicylindrical recess on the distal face of the astragalus. There is a strong possibility that mechanical advantage on the tuber calcis could only be achieved with the foot in a digitigrade pose, as is indicated in the figure reconstruction of Bonaparte¹⁶, and unlike the modern crocodile. We believe it is unlikely that the antero-posterior-deepened calcaneum-astragalus joint in the MPM ankle could be elaborated into the CR condition, although Brinkman⁴ has described an evolutionary pathway which can derive the CR ankle from a CN ancestor. We disagree with this solution, as it is difficult to match homologies of the detailed structures in both types. The origin of the CR ankle must remain obscure for the present, nor do we believe that any convincing AM-R ankle has been described to date. The difficulty in this respect is that many of the AM ankles described in the literature^{2-5,13,14,20} have interlocking processes arising from both calcaneum and astragalus. Therefore, if a process arising from the calcaneum seems larger than any arising from the astragalus, the temptation would be to regard it as representing the CR condition. This arbitrary technique does not identify true homologies and can be refuted in the descriptions of the ornithischian and coelurosaurian ankles given by Cruickshank, who notes² that in the former "The astragalus-calcaneal joint is a series of interlocking processes...". In the coelurosaur ankle a less obvious but similar situation was also illustrated (ref. 2, Fig. 18). These two ankles are of AM-N type, and it thus follows that any ankle of AM morphology which has the calcaneum fitting up against the astragalus laterally must be of CN derivation. Therefore, the assignment of *Vulcanodon*, and by association the sauropods, to the CR condition is wrong^{13,14} and, whatever the relationship of the Ornithosuchidae to other groups, the Carnosauria are probably not of AM-R type either². The ankle of *Allosaurus*²⁰ is essentially similar to that of *Syntarsus*². Although the astragali and calcanea of dinosaurs show a variable pattern of pegs and sockets, these do not function in any way like those of CN and CR ankles. The shared similarities of all AM ankles are so overwhelming that we must assume that this pattern arose once only. The dinosaurs that have been described as having AM-R ankles are really of CN-derived type. If the AM-R ankle existed, it should have the calcaneum-astragalus relationship illustrated by Brinkman (ref. 4, Fig. 8D), with the calcaneum fitting into a notch or recess on the distal face of the astragalus, and not fitting into a recess on the lateral side of the astragalus as is implied elsewhere^{2,5} (Fig. 2, CR). In the CR ankle the calcaneal articulation is distal, as in *Riojasuchus*^{2,16}, and not lateral. Any AM-R ankle must also have the same relationship of astragalus and calcaneum.

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Glutamate stimulates inositol phosphate formation in striatal neurones

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The major excitatory amino acids, glutamate (Glu) and aspartate (Asp), are thought to act at three receptor subtypes¹ in the mammalian central nervous system (CNS). These are termed quisqualate (QA), *N*-methyl-D-aspartate (NMDA) and kainate (KA) receptors according to the specific agonist properties of these compounds revealed by electrophysiological studies^{1,2}. Although Glu has been shown to stimulate cyclic GMP formation in brain slices³, direct regulation of second messenger systems (cyclic AMP, Ca²⁺ or inositol phosphates) subsequent to activation of excitatory amino-acid receptors, has not been extensively studied. Here we demonstrate that in striatal neurones, excitatory amino acids, but not inhibitory or non-neuroactive amino acids, induce a three- to fourfold increase in inositol mono-, di- and triphosphate (IP₁, IP₂, IP₃) formation with the relative potency QA > Glu > NMDA, KA. The Glu-evoked formation of inositol phosphates appears to result principally from actions at QA as well as NMDA receptors on striatal neurones. Our results suggest that excitatory amino acids stimulate inositol phosphate formation directly, rather than indirectly by the evoked release and subsequent actions of adenosine⁴ or acetylcholine⁵.

All experiments were performed on striatal neurones in primary culture, devoid of non-neuronal cell types. The cells were generated from fetal mouse brain and these neuronal cultures provide a good model for the examination of neurotransmitter actions on striatal signal transduction mechanisms⁶. Neurones were raised in the presence of 5 µCi ml⁻¹ ³H-inositol. After 11-14 days *in vitro*, cells were washed, exposed to 10 mM Li⁺ for 10 min to block IP degradation⁷, and putative amino acid neurotransmitters were added for 30 min. The formation of inositol phosphates was followed by sequential chromatography on Dowex-formate columns^{8,18} and liquid scintillation spectrometry.

All putative excitatory amino acids tested evoked increases in inositol phosphate formation (Fig. 1), whereas inhibitory amino-acid transmitter candidates, such as γ-aminobutyric acid (GABA), taurine and hypotaurine, were without effect. Other amino acids with little or no neuroactivity (valine, isoleucine, serine, threonine, methionine, arginine, histidine, tyrosine, tryptophan and proline) did not stimulate inositol phosphate formation. The exposure of striatal neurones to various excitatory amino acids resulted in a dose-dependent and saturable increase in inositol phosphate accumulation (Fig. 2); Glu stimulated inositol phosphate production three- to fourfold; half-maximal

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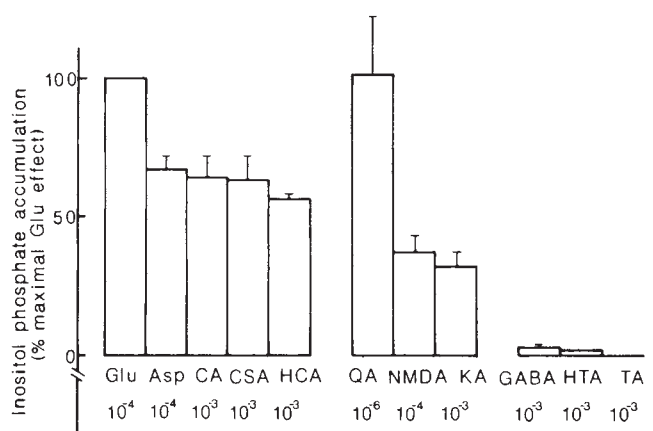


Fig. 1 Effect of amino acids on inositol phosphate formation. Striatal neurones generated from the fetal mouse brain were raised as previously described⁶. Briefly, striata were removed from 14–15-day-old Swiss albino mouse embryos (Iffa Credo, Lyon) and mechanically dissociated with a fire-narrowed Pasteur pipette in serum-free medium. Cells were plated (1×10^6 cells ml^{-1}) in 12-well Costar culture dishes, previously coated successively with poly-L-ornithine ($1.5 \mu\text{g ml}^{-1}$, relative molecular mass 40,000, Sigma) and culture medium containing 10% fetal calf serum. After withdrawing the last coating solution, cells were seeded in serum-free medium ($5 \mu\text{Ci ml}^{-1}$ myo-(^3H)-inositol; NEN) composed of 1/1 mixture of Dulbecco's minimal essential medium and F-12 nutrient (Gibco), and included glucose (0.6%), glutamine (2 mM) and sodium bicarbonate (3 mM), (all from Sigma). In the place of serum, a defined hormone and salt mixture that included insulin ($25 \mu\text{g ml}^{-1}$), transferrin ($100 \mu\text{g ml}^{-1}$), progesterone (20 nM), putrescine (60 μM) and selenium salt (30 nM), (all from Sigma), was added. With this technique, these cultures were immunocytochemically and morphologically defined as purified (>95%) neurones. After 11–14 days *in vitro* the culture medium was replaced with phosphate-buffered saline (composition (mM) NaCl, 137; KCl, 2.7; Na_2HPO_4 , 8; KH_2PO_4 , 1.5; MgCl_2 , 0.5; CaCl_2 , 0.45) and neurones were incubated for 10 min with 10 mM LiCl. Drugs were added at the following concentrations (M): glutamate (Glu) 10^{-4} , aspartate (Asp) 10^{-4} , cysteate (CA) 10^{-3} , cysteine sulphinate (CSA) 10^{-3} , homocysteate (HCA) 10^{-3} , quisqualate (QA) 10^{-6} , NMDA 10^{-4} (the racemic mixture NM(D,L)A was used), kainate (KA) 10^{-3} , GABA 10^{-3} , hypotaurine (HTA) 10^{-3} and taurine (TA) 10^{-3} at 37°C . The reaction was stopped after 30 additional minutes by replacement of the incubation medium by 5% perchloric acid. The phosphates were extracted as described by Bone *et al.*¹⁸ and separated by ion-exchange chromatography as described by Berridge *et al.*⁸. Results are means, expressed as percentage of the response induced by 10^{-4} M Glu, $\pm$ s.e.m. of at least three independent experiments on separate culture preparations, performed in duplicate.

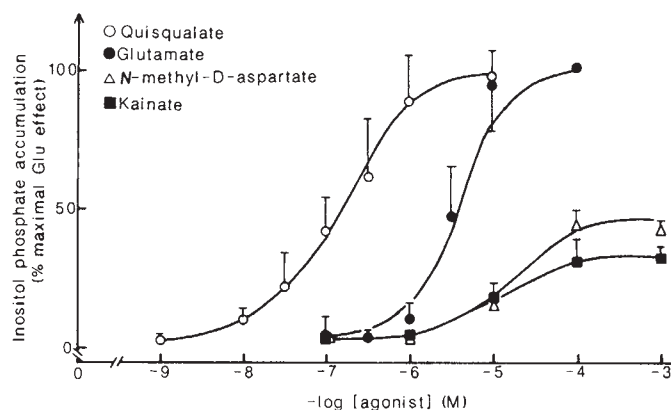


Fig. 2 Dose-response curve for excitatory amino-acid stimulation of inositol phosphate accumulation in neurones. Cells were exposed to increasing concentrations of the indicated amino acids. Results are means, expressed as percentage of the response induced by 10^{-4} M Glu, $\pm$ s.e.m. of three independent experiments on separate culture preparations performed in duplicate.

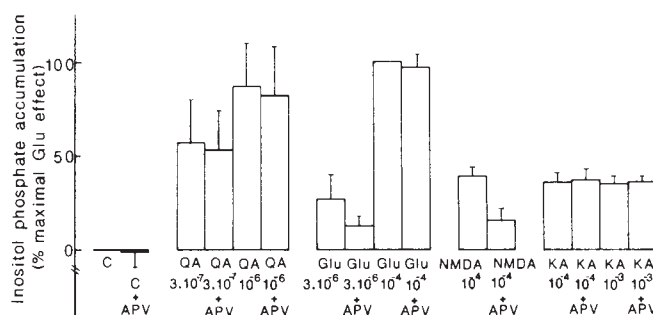


Fig. 3 Effect of NMDA-receptor specific antagonist APV on QA, Glu, NMDA and KA induced inositol phosphate formation. APV ($100 \mu\text{M}$) was added at the same time as LiCl, 10 min before stimulation with the indicated agonists. Results are means, expressed as a percentage of the response induced by 10^{-4} M Glu, $\pm$ s.e.m. of three independent experiments on separate culture preparations, performed in duplicate. The NMDA-induced response in the presence of APV was significantly lower (* $P < 0.05$) than that caused by NMDA alone, as determined with Student's *t*-test.

stimulation (EC_{50}) was obtained with $4 \mu\text{M}$ Glu. QA (EC_{50} $0.16 \mu\text{M}$) was a full agonist, whereas NMDA (EC_{50} $15 \mu\text{M}$) and KA (EC_{50} $10 \mu\text{M}$) stimulated inositol phosphate formation to ~40% of the maximal Glu response. This pharmacological pattern seems to correlate best with the QA rather than KA or NMDA receptor subtypes⁹.

To define further the Glu receptor subtype(s) involved in the stimulation of inositol phosphate accumulation, the effect of 2-amino-5-phosphonovaleate (APV), a selective antagonist at the NMDA receptor⁹, was examined. After the exposure of striatal neurones to $100 \mu\text{M}$ APV for 10 min, the subsequent inositol phosphate response to NMDA was reduced by 56% ($P < 0.05$), while the Glu, QA and KA responses were not significantly affected (Fig. 3). Taken together, these results suggest that, in striatal neurones, excitatory amino-acid stimulation of inositol phosphate formation is mediated by both QA and NMDA receptor subtypes.

In the neostriatum, excitatory amino acids evoke the release of acetylcholine (ACh)⁵, a putative stimulator of inositol phosphate formation in the CNS¹⁰, and striatal neurones (S.W. *et*

al., in preparation). ACh release was completely abolished in the presence of $0.5 \mu\text{M}$ tetrodotoxin (TTX)¹¹. To confirm that the effects of excitatory amino acids were not caused by the release of ACh, inositol phosphate formation was examined in the presence of $0.5 \mu\text{M}$ TTX (Table 1). The responses to QA, Glu, KA and NMDA were unaffected by TTX, suggesting that the stimulation of inositol phosphate evoked by excitatory amino acids is not related to the release of ACh from striatal neurones.

Glu has also been found to stimulate cAMP formation in brain slices¹² via the release of adenosine⁴. However, in striatal neurones, 2-chloro-adenosine ($10 \mu\text{M}$) did not stimulate inositol phosphate formation ($1 \pm 4\%$ of maximal Glu response, $n = 3$), suggesting that the Glu-evoked response is not mediated by the liberation and subsequent action of adenosine. Berridge¹³ has suggested that an interaction may exist between the hormone-sensitive guanylate cyclase and inositol phospholipid hydrolysis. As excitatory amino acids are known to stimulate cGMP accumulation in slices and cell suspensions of the cerebellum³, such an interaction could occur in neurones.

Our findings provide the first evidence for excitatory amino-acid stimulation of inositol phosphate formation in neurones of the CNS. In addition, the data provide a new approach to the examination of excitatory amino-acid receptors via a functional pharmacological response. The high potency (EC_{50} ,

Table 1 Effect of TTX on inositol phosphate response to QA, Glu, NMDA and KA

	Inositol phosphate accumulation (% maximal Glu effect)	
	-TTX	+TTX
Control	0	-4 ± 3
QA (10 ⁻⁶ M)	88 ± 16	91 ± 14
Glu (10 ⁻⁴ M)	100	102 ± 8
NMDA (10 ⁻⁴ M)	39 ± 5	46 ± 15
KA (10 ⁻⁴ M)	38 ± 5	39 ± 5

TTX (0.5 µM) was added at the same time as LiCl, 10 min before stimulation with the indicated agonists. Experiments were carried out as described in Fig. 1. Results are the means, expressed as a percentage of the response induced by 10⁻⁴ M Glu, ±s.e.m. of three independent experiments on separate culture preparations, performed in duplicate.

0.16 µM) and full efficacy of QA in evoking the inositol phosphate response suggest that, in striatal neurones, excitatory amino acids can act principally at QA receptor subtypes in stimulating accumulation of inositol phosphates. However, the selective inhibition of the NMDA response by APV could reflect a separate action of excitatory amino acids at the NMDA subtype.

Results of this study (Table 1) suggest that the inositol phosphate response to excitatory amino acids is neither related to the TTX-blockable release of neurotransmitters (such as ACh) nor to the liberation of adenosine. However, we cannot exclude the possibility that the increase in inositol phosphate formation results from the depolarization induced by direct stimulation of the amino-acid receptors. There is some evidence that K⁺-mediated depolarization results in an increase in inositol phosphate formation in brain slices¹⁴. Similarly, the relationship between the stimulation of inositol phosphate formation by excitatory amino acids and their previously described stimulation of Ca²⁺ uptake in slices of mouse striatum¹⁵ remains unclear, although both effects are more sensitive to Glu than KA, and are resistant to TTX. It is known that cholinergic-induced myopathy shares acute histopathological features with those of the excitatory amino acids¹⁶. As cytotoxic effects in myocytes seem to involve an increased intracellular concentration of Ca²⁺ (ref. 17), the present demonstration of excitatory amino-acid actions on IP₃ formation, known to mediate increases in the intracellular disposition of Ca²⁺ (ref. 7), could be of relevance in elucidation of the neurotoxic mechanisms of these neurotransmitters.

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Inhibition of exocytosis in bovine adrenal medullary cells by botulinum toxin type D

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Botulinum toxins are known to block transmitter release at peripheral cholinergic synapses¹, producing muscular weakness and paralysis. The toxins may also block adrenergic transmission²⁻⁵, although this effect is less well understood¹. The mechanisms by which toxins act are unclear. They are proteins of relative molecular mass ~150,000 and are structurally similar to tetanus toxin. It is generally accepted that a rise in intracellular calcium concentration is sufficient to trigger secretion by exocytosis⁶⁻¹³, but it is not known whether the toxins block secretion by preventing this Ca transient^{14,15} or whether they act downstream from Ca entry by interfering with the process of exocytosis itself. We have attempted to resolve these questions in the case of the adrenergic system by studying the effects of botulinum toxins (types A, B, D and E) on the secretory response of isolated bovine adrenal medullary cells maintained in culture¹⁶. The cells were either challenged with various secretagogues or rendered leaky and challenged directly with Ca buffers. We report here that botulinum toxin type D inhibits secretion in a time- and dose-dependent manner, the results being entirely consistent with the idea that the toxin acts at or near the site of exocytosis rather than at the sites controlling the rise in free Ca.

Chromaffin cells were isolated by protease digestion of thin slices of bovine medullary tissue¹⁷ and cultured at a density of 3 × 10⁵-2 × 10⁶ per ml. Figure 1a shows the effect of incubating the cells for 6 days with various toxins. Only botulinum toxin type D was effective at inhibiting the catecholamine released in response to the physiological secretagogue acetylcholine. Previously boiled toxin type D was without effect. The amounts of toxins used are expressed in terms of mouse lethal doses and correspond to the toxicity, assessed at 3 days, of 50-µl aliquots of the toxins injected into the hind legs of adult mice (20-30 g). Our stock solutions of botulinum type A and E toxins were relatively non-toxic to mice and so the highest doses used corresponded respectively to three and four orders of magnitude less than that used for the botulinum type D toxin, that is, only 10 and 1 mouse lethal doses. The effect of tetanus toxin on catecholamine secretion was also investigated, as inhibition of acetylcholine release from motor nerves by tetanus toxin in some ways resembles the blockade of acetylcholine release by botulinum toxin^{18,19}, and as there is evidence for competition between these two toxins for binding sites in both the peripheral nervous system and synaptosome preparations from the central nervous system²⁰⁻²³. Tetanus toxin had no effect on catecholamine release and the dose-response curve for botulinum type D was unaffected by the presence of tetanus or botulinum type B or E toxins (data not shown). We have been unable to reverse botulinum inhibitions of catecholamine secretion once it is established.

Figure 1a also shows that the total amount of catecholamine associated with the cells after 6 days' incubation increases with the concentration and potency of type D toxin. These data, together with the evidence that very little of the catecholamine of toxin-treated cells is cytosolic (see Fig. 4 and text), argue against the idea that botulinum toxin inhibits secretion by depleting the secretory granules of transmitter. Failure of the toxin to prevent synthesis and storage of acetylcholine at cholinergic synapses has been reported¹. The increased catecholamine content of the cells can be explained in terms of increased catecholamine synthesis or, more probably, in terms of an inhibition of basal as well as evoked secretion. The total catecholamine levels were not increased when cells were incubated with the other toxins (data not shown).

Fig. 1 a, Effect of botulinum and tetanus toxins on catecholamine secretion from cultured bovine adrenal medullary cells. The left-hand ordinate is the amount of catecholamine in the extracellular fluid and is expressed as a percentage of the total catecholamine in the culture dish well. The catecholamine released in response to the acetylcholine challenge in cells incubated in botulinum toxin type A (Δ), B ($\diamond$), D ($\bullet$), E (∇) and for tetanus toxin ($\blacklozenge$) are means ($\pm$ s.e.m.) of three determinations. The extracellular catecholamine concentrations associated with washed, unchallenged cells have been averaged for the different toxin treatments used as they were very similar ($\circ$). The amount of toxin used is expressed in the abscissa as mouse lethal doses (MLD) and correspond to the potencies of 50- μ l aliquots of the incubation culture media. The total amount of catecholamine associated with the cells after incubation with type D toxin is shown on the right-hand ordinate ($\blacksquare$) and is the mean ($\pm$ s.e.m.) of four determinations. Temperature 37 °C. **b**, Effect of time of incubation with botulinum toxin type D on secretion evoked by acetylcholine. Cultured cells were incubated for the periods shown with various concentrations of botulinum toxin type D before being challenged with 2×10^{-4} M acetylcholine for 15 min. The evoked catecholamine released is plotted as the ordinate and is expressed as a ratio of that released by cells of the same age and population incubated without toxin. The data points are means ($\pm$ s.e.m.) of 2-6 determinations and represent accumulated data from 10 experiments. The amounts of toxin used were MLD per 50 μ l of incubation medium; $\bullet$, 2×10^4 ; $\circ$, 2×10^3 ; $\blacksquare$, 2×10^2 ; $\square$, 2×10^1 ; temperature 37 °C.

Methods. Cells were maintained for 6 days in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U ml $^{-1}$ penicillin G, 5 μ g ml $^{-1}$ gentamicin, 5 μ M 5-fluorodeoxyuridine, 5 μ M cytosine arabinoside, and in the presence of various toxins (50 μ l toxin stock in glycerol phosphate buffer per ml cell suspension), after which the plated cells were washed twice with physiological saline containing (mM) NaCl, 150; KCl, 5; HEPES, 10; Ca, 3; glucose, 5; pH 7.3, before being 'challenged' with 0.3 ml of saline either in the absence or presence of 5×10^{-4} M acetylcholine. After 15 min at 37 °C, the solution was assayed for catecholamine. Catecholamine remaining in the cells was released with 0.3 ml saline containing 0.1% Triton and assayed.

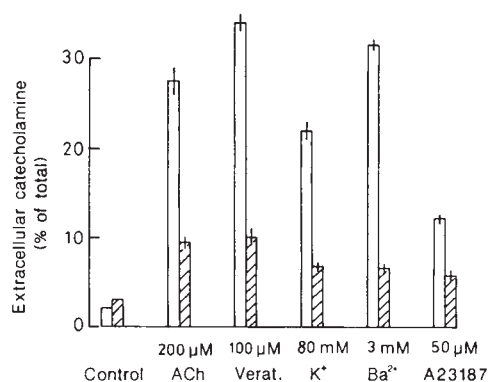
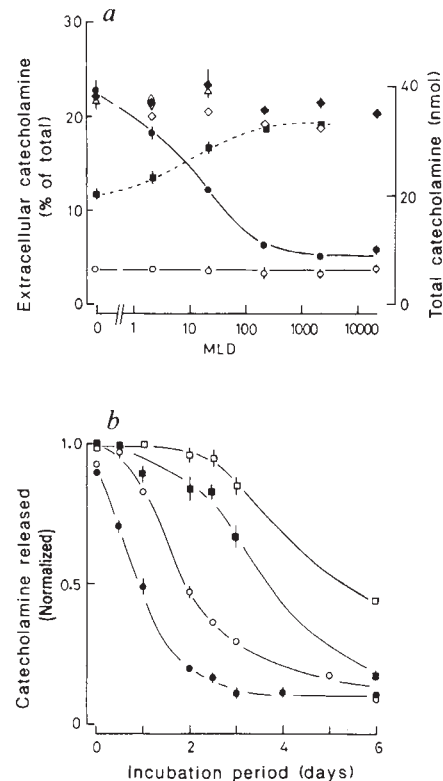


Fig. 2 Effect of botulinum toxin type D on the response of intact cells to various secretagogues. Cells were cultured for 2 days with type D toxin (2×10^4 MLD per 50 μ l), washed and challenged for 15 min at 37 °C, as described in Fig. 1 legend, with the indicated secretagogues (ACh, acetylcholine; Verat., veratridine). The data show the mean of two determinations, the error bars represent the range. Open bars, cells incubated in the absence of toxin; hatched bars, cells incubated in the presence of toxin.

The potency of type D toxin in inhibiting acetylcholine-evoked secretion is also time dependent. Figure 1b shows that toxins differing by a factor of 10 in concentration may be equipotent if the more dilute toxin is exposed to cells for approximately twice as long as the more concentrated toxin. Cells incubated with botulinum toxin type D, when viewed with an inversion microscope, are indistinguishable from untreated cells, plate down normally and exclude trypan blue.

The rise in free Ca necessary to bring about secretion from adrenal medullary cells is generated by Ca entering the cell from

the extracellular medium through Ca channels which are voltage-sensitive and whose action is inhibited by the channel blocker D600; the increased permeability to Ca is triggered by depolarization of the plasma membrane^{6,12,24}. Depolarization can be initiated by various agents, including acetylcholine, veratridine (an alkaloid that opens sodium channels), or directly by raising the extracellular potassium concentration. Figure 2 shows that in all cases the secretory response is inhibited by type D toxin, while measurements of 45 Ca and 22 Na influx suggest that the toxin does not modify Na or Ca fluxes (Fig. 3). The normal D600-sensitive influx of 45 Ca associated with acetylcholine-evoked secretion is not inhibited by the toxin whereas secretion is. Furthermore, the influx of 22 Na which is sensitive to the toxin tetrodotoxin (TTX), and which occurs in the presence of veratridine, is not inhibited by the toxin, whereas secretion is inhibited by both TTX and toxin.

The voltage-sensitive secretory pathway can be bypassed by barium ions, by low concentrations of the Ca ionophore A23187, or by rendering the plasma membrane leaky to Ca buffers using a high-voltage technique^{25,26}. In all cases secretion is inhibited by pretreatment with type D toxin (Figs 2, 4). Blockade of Ca-induced exocytosis in cells preincubated in type D toxin and subsequently rendered permeable by brief exposure to intense electrical fields is particularly interesting because the free Ca to which the secretory apparatus is exposed can be controlled with some precision. Figure 4 shows that there is virtually no Ca-dependent release of catecholamines up to a free Ca concentration of 10 μ M. The simplest interpretation of the data is that botulinum toxin type D inhibits secretion downstream from Ca entry by acting at or near the site of exocytosis itself.

Further experiments are required to determine why bovine adrenal medullary cells are more sensitive to type D toxin than to other toxin types. It is possible that medullary cells possess more receptors for type D toxin, or that all toxins can penetrate medullary cells but only type D can inhibit exocytosis. The

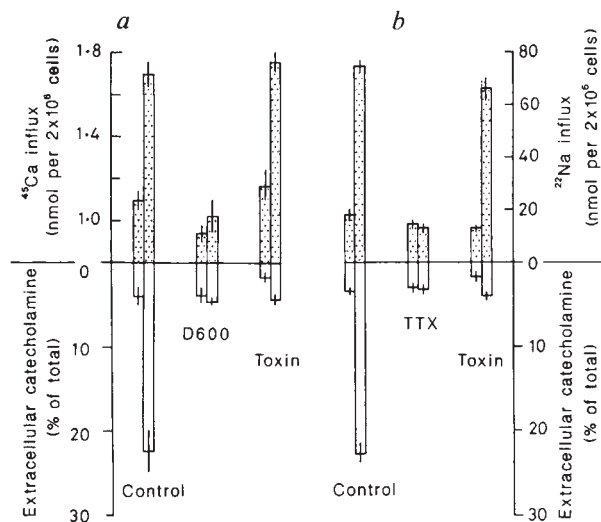


Fig. 3 Effect of toxin type D on ^{45}Ca (a) and ^{22}Na (b) influx. Cells were cultured at $\sim 2 \times 10^6 \text{ ml}^{-1}$ for 5 days either in the absence or presence of botulinum toxin type D (2×10^4 MLD per $50 \mu\text{l}$). The open bars denote the amount of extracellular catecholamine after a 10-min challenge period and the dotted bars denote the ^{45}Ca or ^{22}Na associated with the cells. Data are means of three determinations (error bars are s.e.m.). The second bar of each pair corresponds to measurements from acetylcholine-stimulated cells, the first bar of each pair being data from unstimulated cells.

Methods. a, Plated down cells were washed and incubated for 2 min with 0.2 ml of saline containing 2 mM Ca, either alone or together with 10^{-4} M D600, before being challenged by the addition of 0.2 ml saline containing 2 mM Ca and $4 \mu\text{Ci}$ ^{45}Ca alone or together with 5×10^{-4} M acetylcholine. After 10 min at 37°C the extracellular fluid was removed for counting and catecholamine assay, and the plated down cells were washed five times at 4°C in saline containing 2 mM Ca and 0.5% bovine serum albumin. The cells were then solubilized in saline containing 0.1% Triton, counted and the catecholamine content determined. b, The conditions were the same as for a except that cells were treated with 10^{-5} M TTX instead of D600, were challenged with 10^{-5} M veratridine in place of 5×10^{-4} M acetylcholine, and the 0.2-ml aliquots contained $4 \mu\text{Ci}$ ^{22}Na instead of ^{45}Ca .

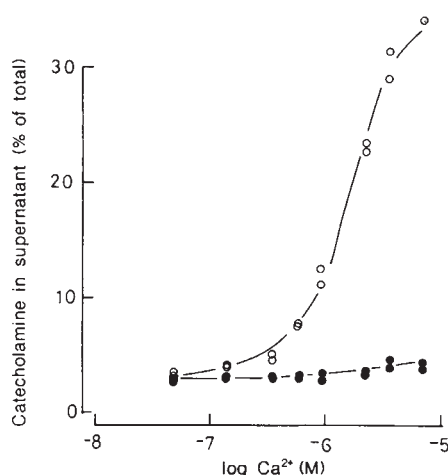


Fig. 4 Effect of botulinum toxin type D on the Ca-dependent secretory response of leaky cells. Cells were cultured in the presence (●) or absence (○) of botulinum toxin type D (2×10^4 MLD per $50 \mu\text{l}$ incubation medium) for 2.5 days in bacteriological Petri dishes. The culture media used were as described in the text but in addition contained 1 mg DNase per 20 ml culture medium to prevent cell aggregation. The cells were washed into a K-glutamate-based medium pH 6.6 containing 5 mM ATP (ref. 26), rendered leaky by 10 exposures of 2 kV cm^{-1} ($\sim 200 \mu\text{s}$), challenged with Ca-EGTA buffers at 28°C , and the catecholamine in the supernatant determined 18 min later.

chromaffin cell should provide an ideal preparation for further investigation into the molecular basis for botulinum poisoning.

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Establishment of idiotypic helper T-cell repertoires early in life

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Immunoglobulin variable-region (V) genes, it is now recognized, do not encode specific receptors for T lymphocytes¹⁻⁴. Classical observations on T-cell expression of immunoglobulin idiotypes^{5,6} had remained unexplained until recent experiments showed that immunoglobulin idiotypes expressed by T lymphocytes in normal mice are absent in cells of the same specificity isolated from donors whose B-cell system has been suppressed by administration of anti- μ antibodies from birth^{7,8}. This observation provided evidence for the 'learning' of T-cell idiotypes from the B-cell/antibody system and, therefore, for the importance of idiotypic network interactions⁹ in the selection of available lymphocyte repertoires before antigenic challenge. Previously described influences of B cells and/or antibodies on the T-helper (T_h) cell compartment¹⁰ would appear to operate at the level of clonal repertoires by complementarities with defined immunoglobulin idiotypes. Other authors, however, had previously shown the striking stability of T-cell idotype expression in chimaeric animals reconstituted with T and B cells originating from donors showing differential idotype expression^{11,12}. We have now investigated this apparent discrepancy and present here results demonstrating that immunoglobulin-dependent selection of T-cell (idiotypic) repertoires only operates for the first 3 weeks of life.

The experimental system previously described has been used throughout these studies⁷. T-helper cells specific for trinitrophenol (TNP)-modified self-antigens (anti-TNP-self T_h) were prepared as before¹³, by intra-tail priming of adult BALB/c mice with TNP-derivatized syngeneic spleen cells and weekly *in vitro* restimulations of the draining lymph node cells with the homologous antigen. After 3 weeks of enrichment, these cells proliferated in response to hapten-modified syngeneic spleen

Table 1 Expression of the F6(51) idiotype by B cells and antibodies in different immune steady states

Immune state*	Absolute frequency of ID ⁺ clonal B-cell precursors†	Serum idiotypic titre‡	Serum anti-idiotypic titre§	ID ⁺ anti-TNP antibody response (µg ml ⁻¹)
Normal	1:2,500	<1:2	<1:2	<0.01
Ab ₁	1:800	1:16	<1:2	0.01–0.1
Ab ₂	<1:14,000	<1:2	1:16,384	<0.01
Ab ₃	1:380	1:4,096	<1:2	150–1,000

* Defined and described in refs 7, 19: Ab₁, Ab₂ and Ab₃, animals immunized with TNP-Ficoll, idiotype (MOPC 460 myeloma protein) and anti-idiotypic (F6(51) monoclonal antibody), respectively, cross-linked to keyhole limpet haemocyanin. The table shows average determinations from many animals per group.

† Summarized from ref. 20. Absolute frequencies are the ratios between the frequency of mitogen-reactive clonal precursors producing ID⁺ immunoglobulin and the total frequency of mitogen-reactive B cells, determined by limiting dilution analysis in the same experiments.

‡ Determined by haemagglutination, using F6(51)-coated red cells²⁰.

§ Determined by haemagglutination, using MOPC 460-coated red cells^{17–19}.

|| Summarized in part from refs 18, 19. Fraction of anti-TNP antibodies, isolated on immunoabsorbent columns, reacting with F6(51) anti-idiotypic.

cells and induced B cells in these populations to extensive proliferation and antibody secretion, revealed by development of immunoglobulin-secreting plaque-forming cells (PFC)^{13,16}. In both types of responses, such T_h showed stringent specificity and the requirements for 'dual recognition' of TNP and Ia^d (refs 7, 13–15). As also shown before⁷, as many as two-thirds of these T_h cells can be inhibited by a monoclonal anti-idiotypic antibody—F6(51)—which recognizes an idiotope on the BALB/c anti-TNP myeloma protein MOPC 460 (ref. 17). Several independent lines of evidence^{7,15} have indicated that such anti-idiotypic antibodies react with clonally distributed receptors on T cells, which determine their paratopic specificity. In short, the inhibition by F6(51) antibodies operates at the level of the responder T_h and not by interactions with the 'target' B cells, these anti-idiotypic antibodies directly induce interleukin-2 production by the appropriate T_h cells, and they fail to inhibit BALB/c T_h cells with several other haptenic specificities. Furthermore, although expression of the T_h idiotype is immunoglobulin allotype-linked⁷, other monoclonal anti-idiotypic antibodies directed to MOPC 460 or to other recurrent anti-TNP idiotypes of BALB/c have no effect on such helper cells (C.M.-A., unpublished data).

The data supporting immunoglobulin-dependent selection of T_h-cell idiotypic specificities led us to investigate their expression in idiotypically manipulated animals. Adult BALB/c mice were immunized with either the anti-TNP myeloma protein carrying the idiotype (M460) or the monoclonal anti-idiotypic antibody F6(51), both cross-linked to keyhole limpet haemocyanin following previously described protocols^{18,19}. These groups of mice, called Ab₂ and Ab₃ respectively, could be shown to produce high levels of auto-anti-idiotypic antibodies (Ab₂) or immunoglobulins complementary to F6(51) (Ab₃) (Table 1). Table 1 summarizes the immune steady states previously analysed in similarly treated animals, where 'Ab₁' and 'normal' denote animals that were not idiotypically manipulated and were either primed with TNP antigen or left untreated. Ab₂ mice are 'suppressed' for the expression of the relevant idiotype on anti-TNP antibodies¹⁹, and display reduced frequencies of clonable B-cell precursors that secrete immunoglobulin carrying that idiotope¹⁹.

In contrast, Ab₃ mice contain very high titres of anti-F6(51) (or idiotype-positive) antibodies and predominantly express this idiotype when immunized with TNP¹⁹. Moreover, these mice contain about 10 times more idiotype-producing B-cell precursors than normal mice²⁰, providing a direct demonstration of the impact of these idiotypic manipulations on the available repertoire of B lymphocytes. To our surprise, however, anti-TNP-self T_h-cell lines prepared from Ab₁, Ab₂ or Ab₃ BALB/c mice (that is, by immunizing either normal, idiotype-suppressed

Table 2 Inhibition by F6(51) anti-idiotypic of the TNP-self-specific T_h activity mediated by T cells prepared in different immune states

Donor of T _h cells*	F6(51) in culture†	Helper (IgM PFC per culture)	Inhibition by anti-idiotypic (%)
Ab ₁	—	11,700	68
	+	3,740	
Ab ₂	—	8,100	55
	+	4,500	
Ab ₃	—	4,600	55
	+	2,070	

TNP-self-specific T_h-cell lines were prepared as described previously¹³ by intra-tail immunizations of BALB/c mice with irradiated (3,300 rad) TNP-derivatized syngeneic spleen cells. The draining lymph node cells were collected 4–15 days thereafter and restimulated during 4 weeks *in vitro* with the homologous antigen. T-helper activity of these lines was assayed by mixing, in 0.2-ml cultures, 4 × 10⁴ TNP-derivatized non-irradiated BALB/c spleen cells with 4 × 10³ anti-TNP-BALB/c T_h cells, and measuring numbers of IgM-secreting PFC in the protein A plaque assay 4 days later¹⁴.

* As in Table 1; no data are available concerning the frequency of TNP-self-specific F6(51)-inhibitable T_h-cell precursors, determined without antigen priming (for example, in lectin-driven limiting dilution systems).

† As indicated, 15 µg ml⁻¹ of purified F6(51) antibody was added at the start of cooperative cultures. Two different methods for affinity purification of F6(51) antibodies from ascitic fluids were used: low pH elution from MOPC 460 columns and pH 6 elution from *Staphylococcus* protein A columns. When tested in parallel, both preparations of F6(51) gave comparable results of inhibition on a weight basis (15, 3 and 0.6 µg ml⁻¹ of antibody resulted in 60%, 35% and 6% inhibition for the protein A-purified antibody, and 63%, 21% and 14% inhibition for the antibody purified on idiotype columns). These two preparations did not contain anti-TNP antibody detectable in haemagglutination assays and were used randomly in these experiments.

or idiotype-primed mice) showed absolutely comparable levels of inhibition by F6(51) antibodies (Table 2). These results apparently contradict the notion that expression of this idiotype by T_h cells results from idiotypic interactions with consequent selection by immunoglobulins and/or B lymphocytes. It could be hypothesized, however, that the mechanisms operating in such idiotypic selection of T_h-cell repertoires act only at critical stages in the development of the mature immune system.

This possibility could be tested because interruption of anti-µ antibody treatments is followed by a rapid reconstitution of the B-cell compartment and normal or supranormal circulating immunoglobulin levels²¹. By suppressing mice from birth with anti-µ antibodies, and interrupting the treatment at various ages, we prepared mice in which the T-cell compartment had developed for various periods of time in the absence of B cell/immunoglobulins. Mice in the various groups were then used to prepare anti-TNP-self T_h cells, which were tested for expression of idiotypes recognized by F6(51) antibodies in functional assays. Two such experiments are shown in Fig. 1. All mice were tested simultaneously at 9 weeks of age. As can be seen, while anti-TNP T_h cells derived from normal BALB/c mice are extensively inhibited by F6(51) antibodies, T_h cells raised in parallel against the same TNP-modified normal BALB/c spleen cells, in mice continuously suppressed with anti-µ antibodies, are not significantly inhibited. Most importantly, mice suppressed with anti-µ for 1 or 2 weeks and allowed to recover thereafter behave like normal untreated mice; in contrast, those in which suppression was maintained for the first 3–4 weeks but then stopped behave as donors that were suppressed for the whole period of the experiment. It would appear, therefore, that interactions with B-cell/immunoglobulin idiotypes that are relevant in the selection of T_h-cell repertoires occur only for the first 3 weeks of life. Mice deprived of the B-cell/immunoglobulin system in this short period of postnatal life did express an abnormal idiotypic T_h repertoire, even though

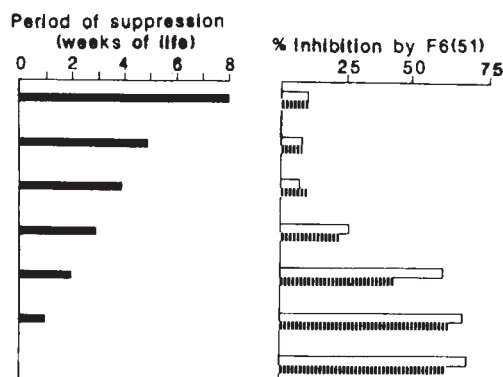


Fig. 1 Two independent experiments showing the extent of idiotypic inhibition in T_h lines derived from different groups of mice suppressed from birth for varying periods by daily injections of rabbit anti-mouse IgM antibodies. The left-hand figure shows the period of suppression while the right-hand figure shows the degree of inhibition by F6(51) antibodies. BALB/c mice were either left untreated or suppressed from birth with daily injections of rabbit anti-mouse IgM antibodies, as described elsewhere⁷. The suppressive regime was interrupted in different groups of mice after the time periods indicated. At 8 weeks, all mice were immunized with TNP-BALB/c cells for the preparation of TNP-self-specific T_h -cell lines, as shown in Table 2. After 3 weeks of *in vitro* enrichment, all T cells were tested for specific T_h activity, using TNP-BALB/c spleen target cells, and for the inhibition of effector T_h activity by addition of F6(51) antibodies (see Table 2). Only one point in the T_h -cell titration is shown (4×10^3 cells per culture) and the control numbers of IgM PFC per culture in the various groups were 4,700–9,080 and 3,400–8,300, respectively, in the first and second experiments.

their B-cell system had been reconstituted for another 4–5 weeks before T_h cells were prepared.

These results suggest that T_h -cell (idiotypic) repertoires are established early in postnatal life and are thereafter quite stable. In the light of these findings, it is not surprising that idiotypic manipulations of adult mice do not lead to detectable alterations in T_h -cell repertoires, as shown above, and that adult T cells do not 'learn' their idiotypes from B cells in chimaeric animals as reported by several authors^{11,12}. This latter phenomenon, together with the allotype-linkage of the control of expression of T-cell idiotypes, has constituted the strongest argument for T lymphocytes bearing V_H -encoded receptor molecules. Our observations help to solve this puzzle and the present incongruities between recent molecular genetics and less recent serological and cellular experiments on T-cell receptor specificities.

The apparent resistance of the T_h -cell repertoires to influences of B-cell/immunoglobulin idiotypes after 3–4 weeks supports the establishment of a stable T-cell repertoire, but on the basis of other selective mechanisms and complementarities, perhaps among the T cells themselves (A. Augustin, personal communication). Furthermore, if on the basis of a recursive organization we expect that T_h -cell repertoires will, in turn, contribute to the selection of available or actual B-cell/antibody repertoires^{22–24}, it could be predicted that mice suppressed with anti- μ antibodies for the first 3 weeks of life will express B-cell repertoires that are variants of those of normal mice. Any speculation on these results should take into account that reactions of anti-idiotypes with T cells are, in our hands, quite exceptional. As noted previously^{7,16}, we have failed to detect any other cross-reactivity of this kind, in extensive tests of a variety of anti-hapten self T_h cells with a large panel of anti-idiotypes, in the appropriate strains. The phenomenon analysed here, if exceptional, nevertheless provides a useful tool in the analysis of systemic regulation.

Finally, if available repertoires of both T and B lymphocytes are implicated in the development of various pathological autoimmune disorders—as suggested by the association of some such diseases with major histocompatibility complex and IgCH

haplotypes^{25,26}—the present observations could be relevant for the understanding of the primary causes of disorder. Thus, immunologically relevant events occurring in perinatal life (such as maternal influences and neonatal infections) could be manifested many years later by such mechanisms operating on the selection of available repertoires. The structural and genetic analysis of T-cell receptors at the molecular level now well under way makes the development of models for studying the regulation of these genes' expression all the more important.

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Irreversible inhibition of Ca^{2+} release in saponin-treated macrophages by the photoaffinity derivative of inositol-1,4,5-trisphosphate

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D-*myo*-inositol-1,4,5-trisphosphate ($InsP_3$) is a putative intracellular second messenger for the mobilization of Ca^{2+} from intracellular stores, in particular, the endoplasmic reticulum^{1–6}. Specific binding sites on the endoplasmic reticulum may participate in the $InsP_3$ -induced release of Ca^{2+} from the Ca^{2+} pool^{6–8}. To examine the specific binding sites on the endoplasmic reticulum, we synthesized an arylazide derivative of $InsP_3$ for photoaffinity labelling; $InsP_3$ coupled to *p*-azidobenzoic acid ($InsP_3$ -pAB) using *N,N'*-carbonyldiimidazole (CDI) was obtained at a 9–11% yield. Here, we report that $InsP_3$ -pAB, but not an arylazide derivative of inositol-1,4-bisphosphate ($Ins(1,4)P_2$), causes the irreversible inhibition of $InsP_3$ -induced release of Ca^{2+} in saponin-permeabilized macrophages. The irreversible inhibition by $InsP_3$ -pAB after photo-irradiation was prevented by a 10-fold excess of unmodified $InsP_3$.

Guinea pig peritoneal macrophages were treated with saponin as described elsewhere^{9,10}. $InsP_3$ was prepared from phosphoinositide (Sigma) by alkaline hydrolysis and purified by TLC

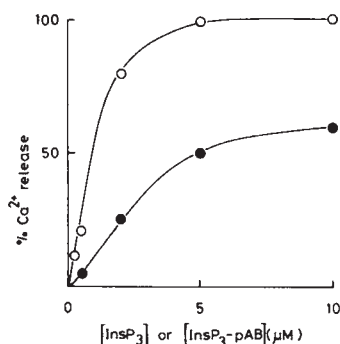


Fig. 1 Dose-response relationship of InsP₃- (○) or InsP₃-pAB (●)-induced Ca²⁺ release. Ca²⁺ was accumulated in saponin-treated macrophages in a solution containing 0.1 M KCl, 20 mM Tris-maleate (pH 6.8), 10 mM NaN₃, 3 mM MgCl₂, 2 mM ATP, 0.12 mM CaCl₂ (containing 2 μCi ml⁻¹ of ⁴⁵Ca²⁺, specific activity 23.8 mCi mg⁻¹; NEN) and 1 mM EGTA for 10 min at 37 °C. The free Ca²⁺ concentration was estimated to be 1.4 × 10⁻⁷ M, on the assumption that the affinity constant of EGTA for Ca²⁺ is 1 × 10⁶ M⁻¹ at pH 6.8 (ref. 3). Ten minutes after the addition of ATP, an aliquot of the mixture was passed through a glass-fibre filter (Whatman GF/C; pore size 1.2 μm) to determine the amount of Ca²⁺ uptake, as described³. At 11 min, the reagent (InsP₃ or InsP₃-pAB) in 1/100 volume of the mixture was added. The Ca²⁺ release is expressed relative to that at 1 min after the addition of 10 μM InsP₃. Two other experiments using different preparations of InsP₃-pAB and cells gave essentially the same results.

on cellulose (Funakoshi, Avicell SF), according to the method of Grado and Ballou¹¹. InsP₃-pAB was prepared under a dim light, by the method of Gottikh *et al.*¹². Briefly, pAB (7,500 nmol; Tokyo Kasei) was mixed with CDI (7,500 nmol; Tokyo Kasei) in 40 μl of dry acetonitrile for 15 min, at room temperature. The solution was then mixed with InsP₃ (750 nmol) in 100 μl of distilled water. The mixture was stirred for 3–4 h at room temperature, and applied to an Avicell SF TLC plate. The chromatography was performed in a solvent containing *n*-propanol, ammonia and distilled water (5:4:1, v/v) for 60–75 min. The reference plate was stained for phosphate ester using molybdate spray reagent¹³. The starting InsP₃ had only one R_F of 0.20, while the InsP₃ mixed with imidazolized pAB (a product of the mixture of pAB and CDI¹²) had R_F values of 0.30, 0.41 and 0.50, in addition to 0.20. Imidazolized pAB had an R_F of 0.79. The two fractions (R_F = 0.20 and R_F = 0.30–0.50) were stripped off the plate and extracted with distilled water. The concentration of InsP₃ was determined by phosphorus assay¹⁴. In a typical preparation starting with 750 nmol InsP₃, 65–85 nmol of InsP₃-pAB was obtained. When the two fractions were dissolved in water and scanned, InsP₃-pAB showed a high absorbance at a wavelength (λ) of 268 nm, with shoulders at 280 and 290 nm (characteristics of an arylazide compound¹⁵) while InsP₃ showed no obvious absorbance. After photo-irradiation for 5 min, the high absorbance of InsP₃-pAB at 268 nm was diminished, indicating the formation of nitrene¹⁵. From the molar extinction coefficient of pAB, the average ratio of pAB to InsP₃ was 1:1.

When saponin-treated macrophages were incubated in a solution containing 0.1 M KCl, 20 mM Tris-maleate (pH 6.8), 10 mM NaN₃, 3 mM MgCl₂, 2 mM ATP and 1.4 × 10⁻⁷ M free Ca²⁺ at 37 °C for 10 min, the Ca²⁺ stored in the cells was 2.2 nmol per 4 × 10⁶ cells (*n* = 10). Application of InsP₃ (10 μM) released ~25% of the stored Ca²⁺ within 1 min. Figure 1 shows the dose-response relationships of InsP₃- or InsP₃-pAB-induced release of Ca²⁺ from saponin-treated macrophages at 1 min after the application of the reagents. InsP₃ extracted from the TLC plate on which the mixture of InsP₃ and imidazolized pAB had been developed, released Ca²⁺ with an ED₅₀ (dose giving half-maximal effect) of 0.9 μM, which is comparable to our previous findings³. InsP₃-pAB also released Ca²⁺, with an ED₅₀ of

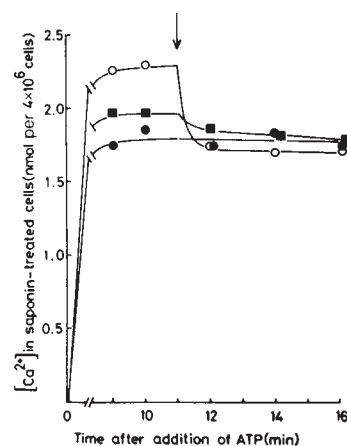


Fig. 2 Effect of InsP₃ (●, 5 μM) or InsP₃-pAB (■, 10 μM) on Ca²⁺ uptake and release. InsP₃ or InsP₃-pAB was included in 5 ml of a solution designed for Ca²⁺ uptake, as described in Fig. 1 legend; 11 min after the addition of ATP (indicated by arrow) 5 μM InsP₃ was added. Open circles show the results of an experiment done in the absence of either InsP₃ or InsP₃-pAB in a solution for Ca²⁺ uptake. Each point represents the mean of four experiments.

2.5 μM, and the Ca²⁺ released by 10 μM InsP₃-pAB was ~60% of that released by the same concentration of InsP₃.

Figure 2 shows the effect of InsP₃ and InsP₃-pAB on Ca²⁺ uptake by saponin-treated macrophages. In the presence of InsP₃, the amounts of Ca²⁺ taken up and stored in the cells were inhibited to the level seen after the addition of InsP₃ to cells which had accumulated Ca²⁺ in the absence of InsP₃. This inhibition by InsP₃ may be due to an increase in Ca²⁺ permeability of the Ca²⁺ pool^{8,16}. Application of InsP₃ after the Ca²⁺ uptake had reached a plateau did not lead to a release of Ca²⁺. InsP₃-pAB also had an inhibitory effect on Ca²⁺ uptake, and the subsequent application of InsP₃ induced a release of Ca²⁺, to a lesser extent. These results indicate that the biological activity of InsP₃-pAB was retained, albeit with a weakened potency.

Saponin-treated macrophages were incubated with 5 μM InsP₃ at 37 °C for 10 min in the absence of ATP, after which InsP₃ was removed by centrifugation. In cells pretreated with InsP₃, we observed that Ca²⁺ uptake was induced by the addition of ATP, and its subsequent release was induced by 5 μM InsP₃, to the same extent as in cells which had not been pretreated with InsP₃ (data not shown), indicating that InsP₃ is reversibly washed out.

We next examined the effect of photo-irradiation on saponin-treated macrophages exposed to InsP₃-pAB and the subsequent release of Ca²⁺ (Fig. 3). Saponin-treated cells were incubated with 10 μM InsP₃-pAB, pAB or InsP₃ at 0 °C for 1 h, and the mixtures were photo-irradiated under a Toshiba FL-20E lamp (λ 270–350 nm) for 10 min at 0 °C. Then the reagent (InsP₃-pAB, pAB or InsP₃) was washed out by centrifugation, and the cells were assayed for Ca²⁺ uptake and subsequent Ca²⁺ release. When saponin-treated cells were photo-irradiated without reagents for 10 min at 0 °C, the Ca²⁺ uptake was inhibited by ~66% compared with the control (that is, the amount of Ca²⁺ stored in non-photo-irradiated cells, 2.2 nmol per 4 × 10⁶ cells was reduced to 1.46 nmol per 4 × 10⁶ photo-irradiated cells). The release of Ca²⁺ induced by 3 μM InsP₃ in the above cells was also inhibited to a similar degree. Ca²⁺ uptake by saponin-treated photo-irradiated cells was inhibited to a greater extent by 10 μM InsP₃-pAB, and the subsequent Ca²⁺ release induced by InsP₃ was also strikingly inhibited (Fig. 3b). On the other hand, the uptake and release of Ca²⁺ in cells which had been incubated with 10 μM InsP₃-pAB but not photo-irradiated, were the same as in the control cells. However, the Ca²⁺ release was inhibited slightly (Fig. 3a). Cells treated with unmodified InsP₃

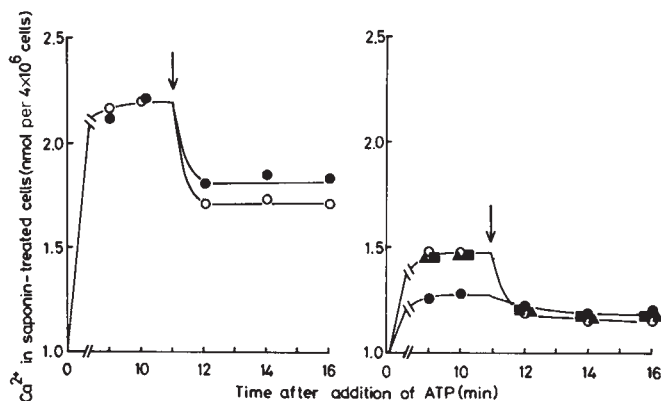
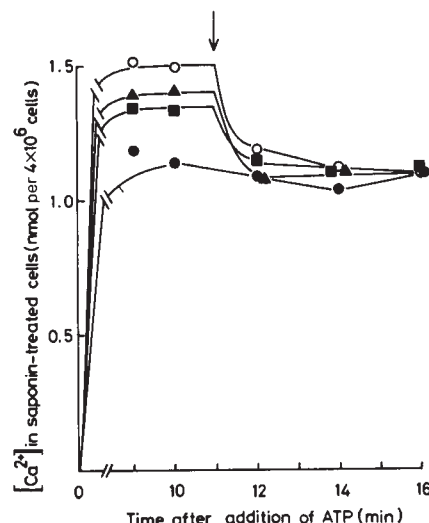


Fig. 4 Specificity of the irreversible inhibition of Ca^{2+} uptake and release in the cells treated with InsP_3 -pAB and photo-irradiated. The incubation, photo-irradiation, and Ca^{2+} uptake and release assays were carried out as described for Fig. 3. The incubation medium contained $10 \mu\text{M}$ InsP_3 -pAB (●), InsP_3 -pAB plus $100 \mu\text{M}$ unmodified InsP_3 (■), $10 \mu\text{M}$ $\text{Ins}(1,4)\text{P}_2$ -pAB (▲), or none of these (○). Two other experiments gave essentially the same results.

Fig. 3 Effect on Ca^{2+} uptake and release of photo-irradiation of cells treated with InsP_3 -pAB. *a*, No photo-irradiation; *b*, photo-irradiated. The incubation medium contained either InsP_3 -pAB (●), pAB (▲), InsP_3 (■), or none of these (○). The data are representative of at least nine experiments.

Methods: Saponin-treated macrophages (2×10^7 cells) were incubated in 1 ml of a solution containing 0.1 M KCl, 20 mM imidazole-HCl (pH 6.8), 1 mM MgCl_2 and 1 mM EGTA, with or without InsP_3 , pAB or InsP_3 -pAB (at a concentration of $10 \mu\text{M}$) for 1 h at 0°C . The mixture was then transferred to a Lab-Tek tissue culture chamber (4802; Miles) and photo-irradiated under a Toshiba FL-20E lamp (wavelength 270–350 nm) at a distance of 10 cm, for 10 min at 0°C . Because the cells have high absorbance, Lab-Tek tissue culture chambers (4802) were used to ascertain the photolysis. Using the chambers, the thickness of the mixture (1 ml) was remarkably reduced to 1–2 mm¹⁵. To assay for Ca^{2+} uptake and release, the mixture was washed twice with a solution containing 0.1 M KCl and 20 mM Tris-maleate (pH 6.8) to remove the reagent, and finally suspended in 1 ml of the solution. Ca^{2+} uptake and release were assayed as described in the legends to Figs 1, 2.



or the same concentration of pAB as that in InsP_3 -pAB, and photo-irradiated, could accumulate Ca^{2+} , and Ca^{2+} release induced by InsP_3 was also observed to the same degree as in the control cells.

We next examined the specificity of the irreversible inhibition of InsP_3 -induced Ca^{2+} release in cells photo-irradiated with InsP_3 -pAB (Fig. 4). First, competition experiments using unmodified InsP_3 were done. When InsP_3 -pAB was allowed to bind to saponin-treated macrophages in the presence of an excess of unmodified InsP_3 ($100 \mu\text{M}$) before photo-irradiation, there was no obvious inhibition of Ca^{2+} release (Fig. 4). Although a slight inhibition of the Ca^{2+} uptake was observed, this may have been the result of InsP_3 in the binding medium being transferred to the solution for Ca^{2+} uptake, because a very high concentration of InsP_3 was included in the binding medium. Furthermore, we synthesized $\text{Ins}(1,4)\text{P}_2$ -pAB, using the same method as for the synthesis of InsP_3 -pAB. The R_F of $\text{Ins}(1,4)\text{P}_2$ -pAB was 0.43, while that of $\text{Ins}(1,4)\text{P}_2$ was 0.26; 13% of $\text{Ins}(1,4)\text{P}_2$ was obtained as $\text{Ins}(1,4)\text{P}_2$ -pAB, and 1 mol of pAB was estimated to be coupled to 1 mol of $\text{Ins}(1,4)\text{P}_2$, as determined by the absorbance of $\text{Ins}(1,4)\text{P}_2$ -pAB at 268 nm. Although Ca^{2+} uptake was slightly inhibited by $\text{Ins}(1,4)\text{P}_2$ -pAB, Ca^{2+} release was evident in photo-irradiated cells that had been treated with the reagent.

In the present study, we obtained three types of InsP_3 -pAB having different R_F values on cellulose TLC. However, because only a limited amount of InsP_3 -pAB was obtained, we used a mixture of the three types and could not, therefore, verify the site of attachment of pAB to the InsP_3 molecule. InsP_3 -pAB caused an irreversible inhibition of Ca^{2+} uptake and release in saponin-treated macrophages after photo-irradiation, which

indicates that InsP_3 can bind irreversibly to the binding site on the InsP_3 -sensitive Ca^{2+} pool in macrophages.

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Distribution of factor VIII mRNA and antigen in human liver and other tissues

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The cellular site of synthesis of factor VIII (FVIII:C; anti-haemophilic factor) has long been sought¹. Previous studies suggested the liver as a major site of synthesis, but extrahepatic sources such as spleen and lung have been implicated²⁻⁶. Using an immunoradiometric assay (IRMA), we recently localized factor VIII antigen (FVIII:Ag, formerly FVIII:C_{Ag}), to whole perfused guinea pig liver and spleen, and to isolated hepatocytes, with lesser or trace amounts in other tissues⁷. Using an immunohistological technique, Stel *et al.*⁸ detected FVIII:Ag in normal human liver sinusoidal endothelial cells, while Exner *et al.*⁹ detected FVIII:Ag by IRMA in extracts of human lymph nodes, lung, liver and spleen. The localization of antigen in tissues does not, however, distinguish sites of factor VIII synthesis from those of storage, and such experiments are subject to misinterpretation due to entrapment of plasma factor VIII in tissues. The recent cloning of the human factor VIII gene¹⁰⁻¹² provides hybridization probes for the detection of factor VIII messenger RNA in cells, thus directly determining sites of synthesis. During complementary DNA cloning, we detected factor VIII mRNA in liver¹⁰, and it has been localized by others in liver and placenta¹² and in liver and kidney¹³. In the present study, we detected factor VIII mRNA in isolated human hepatocytes, in spleen and in numerous tissues including lymph nodes and kidney, but not in white blood cells or cultured endothelial cells. We also found that the factor VIII, factor VII, factor IX and protein C antigens in liver are predominantly localized in hepatocytes, while very little von Willebrand factor antigen (vWF:Ag, formerly FVIII_RAg) is detectable in this organ.

Normal human tissues were obtained from organ donors and were immediately preserved in liquid nitrogen for RNA preparation^{14,15}. Liver cell suspensions were prepared from fresh tissue, and hepatocyte and sinusoidal cell fractions were isolated (Fig. 1). Endothelial cells were cultured from human umbilical veins¹⁶ and were provided by David Stern. Haemophilic tissue samples were obtained at splenectomy for idiopathic thrombocytopenic purpura (spleens); 1 h postmortem (liver); and from an 18-week-old fetus (liver, brain, heart and placenta).

The various cell types were tested for the presence of factor VIII mRNA by Northern blot hybridization¹⁷ and RNase protection mapping^{18,19}, using the cloned factor VIII gene^{10,11} as the source of probes. We had previously used the RNase mapping technique to determine the 5' start site of factor VIII mRNA derived from human liver and AL-7 hybridoma cells¹¹. In our hands, the RNase mapping procedure was more sensitive than Northern blotting, and therefore served as our primary method of RNA detection. For these experiments, ³²P-labelled anti-messsage-strand RNA was synthesized from a 1.2-kilobase (kb) *Sst*I fragment of the human genome spanning the 5' end of the factor VIII gene¹¹. On annealing to samples containing factor VIII mRNA and after digestion by single-strand-specific nuclease RNase A and T1, this probe is reduced to a fragment of 185 bases and a weaker 187-base species, which serve to map the 5' end of factor VIII mRNA (Fig. 2a)¹¹. To further verify our results, many of the RNA samples were also subjected to RNase mapping with probes synthesized from the middle (exon 14) and 3' end (exon 26) of the factor VIII gene (examples in Fig. 2b), and to Northern blot analysis (not shown). In each case, bands of the predicted size were observed (see Table 1).

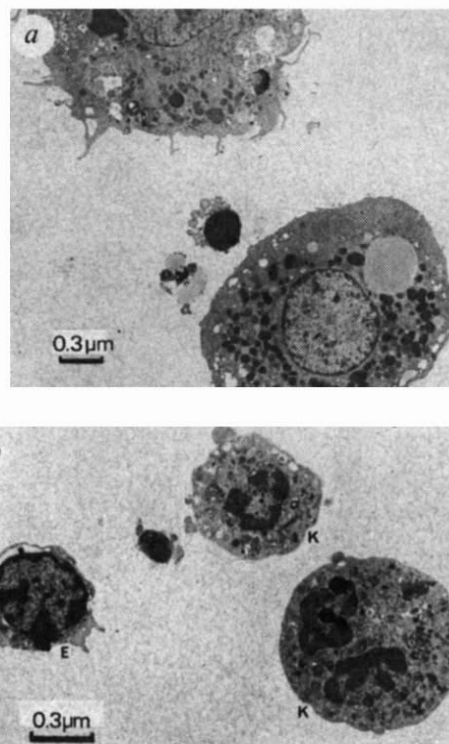


Fig. 1 Electron microscopic sections of human hepatocyte (a) and sinusoidal cell (b) fractions showing an endothelial cell (E) and Kupffer cells (K). Normal human liver was obtained from organ donors. Unfrozen peripheral wedge-shaped slices were rapidly perfused through large vessels on their cut surface with a Ca^{2+} -free Hank's balanced salt solution (HBSS) at 37 °C to remove blood, and were further perfused with HBSS buffer containing 0.05% collagenase at 37 °C for 20 min. The disaggregated liver cell suspensions were separated by differential centrifugation into hepatocyte and sinusoidal (endothelial plus Kupffer) cell fractions⁷. To overcome contamination of the sinusoidal cell fraction by hepatocytes, this fraction was further treated with pronase. Following this, the 'hepatocyte' fraction contained 84% hepatocytes and 16% sinusoidal cells; the 'sinusoidal' fraction contained no hepatocytes, 85% sinusoidal cells and 15% debris, classified according to electron microscopic morphology of 100 cells per fraction.

Factor VIII mRNA was detected primarily in whole normal liver and isolated hepatocytes, in spleen and lymph nodes, and in lesser amounts in pancreas, kidney, muscle and placenta (Fig. 2, Table 1). Significantly, factor VIII mRNA was not detected in bone marrow, peripheral blood lymphocytes or buffy coat white blood cell preparations. The negative results with circulating blood cells serve as a control against a spurious signal in liver or spleen tissue deriving from trapped blood cells. Factor VIII mRNA was detected in two continuous cell lines: low concentrations were present in the hepatocyte-derived Alexander cells, and more substantial quantities were found in the human T-cell hybridoma line AL-7 (ref. 10). (AL-7 cells were the source of the RNA used in the cloning of factor VIII cDNA; however, we detected no factor VIII mRNA in several additional T- and B-cell hybridomas or in various leukaemia-derived cell lines¹⁰. A detectable signal was not seen with Alexander cells when RNA from a preparation of poorer quality was used¹¹.) Note that neither factor VIII activity nor FVIII:Ag could be convincingly detected in supernatants of AL-7 or Alexander cells; this probably reflects the greater sensitivity of the RNA detection methods, although we cannot exclude the possibility that these cells synthesize factor VIII mRNA, but do not secrete the protein.

To further localize the cellular sites of synthesis, we estimated the levels of factor VIII mRNA and antigen in separated liver hepatocyte and sinusoidal cell fractions. While both factor VIII mRNA and antigen were readily detectable in hepatocytes, only

Table 1 Factor VIII mRNA in human tissues and cells

Tissue/cell	Factor VIII mRNA	Haemophilic tissue	Factor VIII mRNA
Whole liver	++	Liver, Case 1	++
Hepatocyte fraction	+	Spleen, Case 1	++
Sinusoidal fraction	-	Spleen, Case 2	++
Spleen	++		
Lymph node	+++	Fetal heart	++
AL-7 cell line	++	Fetal liver	+
Pancreas	+	Fetal lung	-
Kidney	+	Fetal brain	-
Alexander cell line*	+		
Placenta (full term)	+		
Muscle	+		
Thymus	-		
Bone marrow	-		
Peripheral lymphocytes	-		
Buffy coat	-		
Endothelial cells†	-		
U937 cells‡	-		

The results of RNase mapping experiments (Fig. 2) are tabulated, with positive results for factor VIII mRNA scored as + for lowest and +++ for highest signal intensity.

* Human hepatoma, ATCC CRL 8024.

† Human umbilical vein endothelial cells at low passage, either with or without endotoxin stimulation.

‡ Human histiocytic lymphoma with monocyte-like morphology, ATCC CRL 1593.

trace amounts of FVIII: Ag and no mRNA were detected in the sinusoidal cells (Fig. 2a, lanes 6, 27, 28 and Fig. 3b). (Low levels of factor VIII mRNA in sinusoidal cells could have escaped detection due to the small amounts of RNA recovered from this liver cell fraction.) Although low-level synthesis in the sinusoidal cells is not ruled out, it cannot be quantitatively significant in maintaining plasma factor VIII activity.

Figure 2 also includes RNA derived from spleen and liver of two patients with severe haemophilia A and from a haemophilic fetus. Factor VIII mRNA is present at apparently normal levels in all these tissue samples, suggesting that their particular haemophilic phenotype is not due to defective RNA transcription or stability. By analogy to the genetic defects observed in thalassaemia, it is reasonable to postulate that haemophilia may be caused by various molecular defects affecting RNA or protein production and maturation. Thus, in only some cases of haemophilia would the absence of factor VIII mRNA be expected.

Estimates of mRNA levels were obtained by comparing the autoradiographic signal intensity of protected factor VIII bands and of RNase-protected bands using dilutions of human liver RNA and a radiolabelled serum albumin probe. The extremely low levels of circulating factor VIII (200 ng ml⁻¹ or ~10⁻⁶ M of serum albumin) are reflected in the levels of mRNA. Based on reported levels of serum albumin mRNA (7% of human liver poly(A)⁺ RNA²⁰), factor VIII mRNA accounts for ~0.001% of liver, spleen and AL-7 hybridoma cell poly(A)⁺ RNA. A somewhat higher mRNA level was seen in the single para-aortic lymph node that we examined. In a previous report⁷, we noted comparable amounts of FVIII: Ag per gram of liver and spleen tissue. Because of relative organ size, however, about 5–10 times more factor VIII might be synthesized in liver than in spleen.

Figure 3a demonstrates the presence of readily detectable quantities of the antigens of the vitamin K-dependent factors VII, IX and protein C in perfused whole liver. A significant but lesser quantity of factor VIII antigen, and only negligible quantities of von Willebrand factor, were detected. After cell separation (Fig. 3b) there was variable recovery of antigen; nevertheless, it is clear that all four detectable antigens (IX, VII, protein C and VIII) were predominantly localized in the hepatocyte fraction, with negligible or only background quantities in the sinusoidal cell fraction. vWF: Ag was not detected in either cell

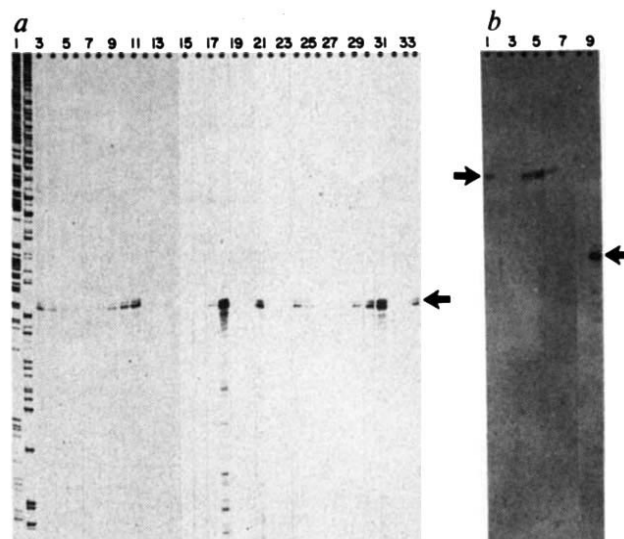


Fig. 2 Detection of factor VIII mRNA by RNase protection mapping. Autoradiographs of 6% polyacrylamide/7M urea gels are shown. **a**, A uniformly labelled, anti-sense RNA probe was synthesized from a 1.2-kb *Sst*I fragment of genomic DNA containing the initial portions of the factor VIII coding region (exon 1) and 5' flanking sequences¹¹. Following hybridization of the labelled probe to sample RNA, digestion with single-strand-specific RNase yielded 185- and 187-base fragments (marked by arrow) containing the 5' end of the factor VIII message. Lanes 1, 2, a known DNA sequencing ladder used as a size standard. Lanes 3–34, the results of RNase protection reactions containing ~10 µg per lane (unless otherwise noted) of unlabelled cell and tissue poly(A)⁺ RNA (all cells were of non-haemophilic adult human origin unless otherwise noted): lane 3, AL-7 hybridoma cells; 4, liver sample 1; 5, liver sample 2; 6, isolated hepatocytes; 7, haemophilic liver; 8, haemophilic spleen 1; 9, haemophilic spleen 2; 10, spleen 1; 11, spleen 2; 12, buffy coat white blood cells; 13, mouse T-lymphoma cell line S49 (control); 14, no RNA (control); 15, thymus; 16, U937 'monocyte-like' cell line; 17, placenta; 18, lymph node; 19, buffy coat cells (sample 2); 20, peripheral blood lymphocytes; 21, liver sample 3; 22, umbilical vein endothelial cells (endotoxin-stimulated); 23, umbilical vein endothelial cells (unstimulated); 24, kidney; 25, pancreas; 26, bone marrow; 27, isolated sinusoidal endothelial cells (~3 µg); 28, isolated hepatocytes; 29, Alexander hepatoma cells; 30, muscle; 31, haemophilic fetal heart; 32, haemophilic fetal brain; 33, haemophilic fetal lung; 34, haemophilic fetal liver. Overexposures to detect faint signals led to the appearance of incomplete digestion products smaller than 185 bases in some lanes. **b**, Examples of RNase protection mapping performed as in **a** but with RNA probes prepared from two different regions of the factor VIII gene. In lanes 1–7, a labelled, anti-sense RNA probe of 490 bases was synthesized from a 270-base *Xmn*I fragment containing exon 14 (plus vector sequences), which protects a 270-base fragment (marked by arrow) of factor VIII RNA from appropriate cells. Lanes 8 and 9 are examples of results obtained with a 420-base probe synthesized from a 211-base *Bam*HI fragment containing exon 26 (plus vector sequences), which protects a 211-base fragment (arrow) in preparations from cells producing factor VIII RNA. Sources of poly(A)⁺ RNA (10 µg) are: 1, liver; 2, buffy coat cells; 3, U937 cells; 4, isolated hepatocytes; 5, spleen; 6, lymph node (2 µg); 7, no RNA (control); 8, buffy coat cells; 9, liver. RNA was prepared as described elsewhere^{14,15}. The genomic factor VIII fragments¹¹ were ligated into pSP64 and ³²P-RNA probes were synthesized as described previously^{18,19}. For detailed reaction procedures see ref. 11.

fraction. Von Willebrand factor is known to be synthesized by vascular endothelial cells²¹ but is clearly not localized in hepatic endothelial cells, which are morphologically different from their vascular counterparts. Localization of the vitamin K-dependent factor antigens (IX, VII and protein C) to the hepatocyte fraction confirms and extends previous studies^{22–24} and lends credence to the factor VIII data. The varying levels of antigen in whole liver presumably reflect varying rates of synthesis and export. Factor VII has the shortest half-life of the factors and may be rapidly exported from the hepatocyte.

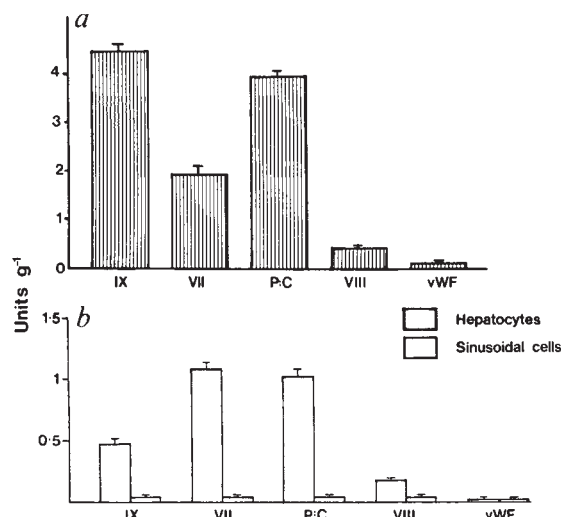


Fig. 3 Localization of coagulation protein antigens in homogenized human liver (a) and in isolated liver cell fractions (b). Each value represents the mean $\pm$ s.e.m. of three samples from different donors. Aliquots of human liver were perfused with HBSS buffer and homogenized with protease inhibitors⁷. Isolated liver cell suspensions were obtained as described in Fig. 1 legend, and homogenized. Coagulation protein antigens from whole and isolated liver cells were measured in the supernatants by sensitive radiometric assays: factor IX (IX)³⁶, factor VII (VII)³⁷, protein C (P-C)³⁸, factor VIII (VIII)³⁹, and von Willebrand factor (vWF)⁴⁰. Antigens are expressed as units per g wet weight of whole liver or isolated cells. One unit is the quantity of antigen present in 1 ml of pooled normal plasma ($n=20$) for each protein.

The vitamin K-dependent factors are synthesized exclusively in the liver and by macrophages, in contrast to factor VIII, for which many extra-hepatic sites are identified here—this may account for the relatively lower concentration of FVIII:Ag found in whole liver. The data presented here demonstrate the localization of FVIII:Ag and mRNA to whole human liver, spleen and isolated hepatocytes, confirming a previous study in guinea pigs⁷. In addition, by detecting factor VIII mRNA, we have shown that although factor VIII is synthesized mainly in the liver, spleen and lymph nodes, synthesis also occurs in many extra-hepatic sites in the adult and in the fetus. Furthermore, the predominant liver cell responsible for synthesis is the hepatocyte. This result is in contrast to those of Stel *et al.*⁸, who reported localization of FVIII:Ag to sinusoidal endothelial cells only; this may have been an artefact of their immunohistological techniques, or due to storage of the antigen in sinusoidal endothelial cells (liver lipase, for example, is manufactured in hepatocytes and stored in the sinusoidal cells before release^{25,26}).

The fact that the concentration of factor VIII does not decrease in human chronic liver disease has led to the belief that the hepatocyte is not the site of factor VIII synthesis. However, many factors other than synthesis are abnormal in this situation, such as catabolism, clearance and the acute-phase response²⁷. In particular, levels of protein C, a potent inactivator of factor VIII, would diminish with liver dysfunction. After acute destruction of hepatocytes by toxins in the rat, the concentration of factor VIII fell rapidly²⁸. From the results reported here, it is now reasonably certain that the hepatocyte is a major source of plasma factor VIII but that extra-hepatic sites of synthesis also exist. The specific cell type(s) producing factor VIII in other tissues remains uncertain. An attractive hypothesis would be that the endothelial cell, a universally distributed cell type already known to produce von Willebrand factor, is responsible. However, previous studies^{29–31} failed to detect factor VIII:Ag or coagulant activity in umbilical vein endothelial cells. In this study no factor VIII in RNA could be detected in these cells either with or without endotoxin stimulation. (Low levels of apparent factor VIII-bypassing activity, that is, factor IXa-mediated factor X activation, have been observed in endotoxin-stimulated endothelial cells; D. Stern, personal communication.)

Conceivably, endothelial cells in organs other than the umbilical cord may produce factor VIII.

The results we have obtained for distribution of factor VIII mRNA in tissues broadly agree with the distribution of FVIII:Ag reported previously^{7,9,32} and do not correlate directly with vascularity (for example, lymph nodes were more strongly positive for factor VIII mRNA than kidney) but rather suggest that a range of differentiated cell types produce factor VIII. It may be that widespread production of this unstable co-factor for intrinsic pathway coagulation is essential to protect many organs from internal haemorrhage. Haemophiliacs frequently bleed into their muscles, joints and kidneys. The negative result that we obtained for bone marrow is in accord with the failure of bone marrow transplantation to correct canine haemophilia³³. However, the finding of factor VIII mRNA in lymphoid tissue agrees with transplantation studies showing correction of canine haemophilia by such tissue³⁴. That the cell type in lymph nodes and spleen which produces relatively large amounts of factor VIII mRNA may be a differentiated lymphoid cell calls to mind the case of a haemophilic patient who developed acute lymphatic leukaemia³⁵. When the leukaemia presented, the concentration of factor VIII in the patient rose to normal levels, but declined to zero on remission. In a subsequent relapse the factor VIII level again rose. It therefore seems worth directing future efforts at precisely locating extra-hepatic sites of factor VIII synthesis towards lymphoreticular cell lines and other organ-specific differentiated cell types.

We thank the following for providing human tissues and cell lines: Mr O. Fernando (Royal Free Hospital) who provided normal human livers, Peter Jones (Newcastle Royal Infirmary), Reuben Mibashan (King's College Hospital), and Bradford Baer, Patrick Gray, Axel Ullrich, David Martin, and Lloyd Svedersky of Genentech. The adult haemophilic liver sample was obtained 1 h postmortem; it had been the patient's express wish when dying that his body should be donated to research. The umbilical vein endothelial cells were provided by David Stern of Columbia University, who we also thank for permission to include his unpublished results. Dr Alan Payne and Mr R. Legge of the MRC Toxicology Unit, Carshalton, assisted in setting up the liver perfusion technique. Jeanne Arch and Alane Gray assisted in preparing the manuscript and figures. D.K. and J.A.S. were supported by the Wellcome Trust. Speywood Laboratories and Genentech, Inc. provided financial assistance.

Note added in proof: Recently, haemophilia has been cured in a human patient by transplantation of a normal liver⁴¹, thus dramatically confirming our main conclusion.

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Ultrastructural localization of factor VIII procoagulant antigen in human liver hepatocytes

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Factor VIII is generally believed to circulate in blood as a multimeric complex of two glycoproteins which are physiologically and immunologically distinct. One component of the factor VIII complex is factor VIII procoagulant activity (FVIII:C) which is associated with factor VIII procoagulant antigen (FVIII:Ag, formerly FVIII:CAg). The second, larger unit of the complex is factor VIII/von Willebrand factor (vWF:Ag, formerly factor VIII-related antigen or FVIII:RAg). FVIII:C has anti-haemophilic

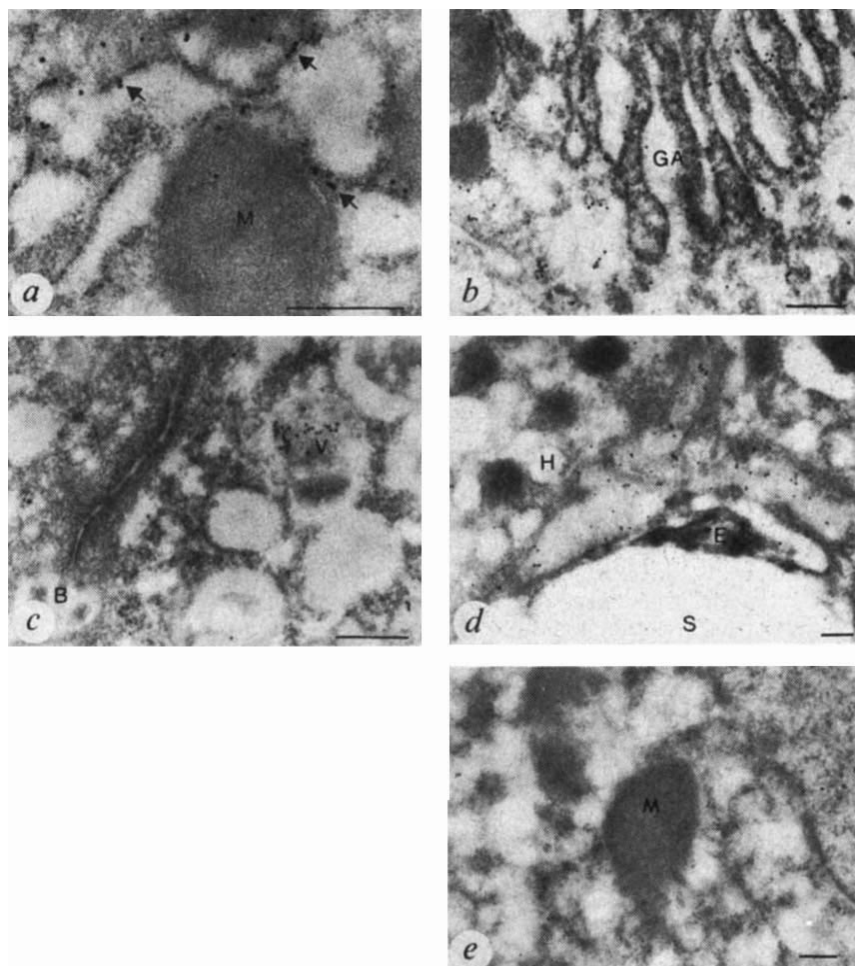
activity and is defective or deficient in patients with classical haemophilia, and vWF:Ag is absent in patients with von Willebrand disease¹⁻³. FVIII:Ag was demonstrated recently in endothelial cells lining hepatic sinusoids, by using immunoperoxidase staining and light microscopy^{4,5}, whereas biochemical data had indicated its presence predominantly in the hepatocyte fractions and in lesser amounts in endothelial cells^{6,7}. Moreover, recent hybridization experiments detected FVIII:C messenger RNA in liver⁸ and kidney⁹ tissues. Despite several efforts, the cells responsible for FVIII:C synthesis have not been unequivocally identified. Here we use protein A-gold complex labelling to demonstrate the ultrastructural localization of FVIII:C in human liver cells; the results indicate that hepatocytes may synthesize FVIII:Ag.

Studies were performed with previously described CLB-CAg A monoclonal antibody specific for FVIII:Ag^{4,10}. Human liver biopsy tissue was obtained from seven young patients with no significant liver disease and from one haemophilic patient. After biopsy, the liver tissues were immediately fixed and processed for low-temperature embedding in Lowicryl K4M resin according to the procedure^{11,12} described in Fig. 1 legend.

In normal liver sections incubated with CLB-CAg A then treated with protein A-gold complex, immunoreactive sites were found in all hepatocytes and sinusoidal endothelial cells examined. Within the hepatocytes, gold particles were observed on the attached ribosomes segregated in the rough endoplasmic reticulum (RER). In the same hepatocytes we observed totally labelled, partially labelled and unlabelled cisternae. Immunolabelled RER often delineated the mitochondria (Fig. 1a). Immunoreactive material was also observed within the saccules and on the membranes of the Golgi apparatus

Fig. 1 Protein A-gold labelling of thin sections of human liver prepared for electron microscopy in Lowicryl K4M and incubated with CLB-CAg A antibody. Scale bars represent 0.4 μ m. *a*, Immunolabelling of rough endoplasmic reticulum of hepatocytes, showing the mitochondria (M). Note that the label accurately delineates the ribosomes (arrows). *b*, Labelling of the total Golgi apparatus (GA). Immunoreactive material is present throughout all the cisternae. *c*, Specific staining of the vacuoles (V). The density of label within the same vacuole is not uniform. The bile capillary duct (B) does not show any labelling. *d*, Extracellular labelling between the microvilli of hepatocytes (H) and endothelial cells (E) of the sinusoid (S). *e*, Negative control, comprising section of liver from a haemophilic patient.

Methods. Small blocks of liver tissue were fixed immediately after biopsy in phosphate-buffered 1% glutaraldehyde pH 7.4, for 1 h at 20 °C. Dehydration through an ethanol series at 0 °C, -5 °C and -20 °C, and infiltration with Lowicryl K4M, were done according to the manufacturer's recommendation (J.B.E.M., Canada). Polymerization was carried out under ultraviolet light (365 nm) at -20 °C for 48 h, then at room temperature for 24 h. The sections were cut with a glass knife and processed as follows for protein A-gold labelling on 300-mesh nickel grids carrying carbon-coated Formvar. The grids were floated on drops of phosphate-buffered saline (PBS) pH 7.4 for 5 min at room temperature, transferred to CLB-CAg A monoclonal antibody (freeze-dried ascitic fluid, 60 μ g ml⁻¹) diluted in PBS, and then maintained at 4 °C in a humidified chamber for 24 h. They were then jet-washed in PBS and floated on the protein A-gold solution (diluted 1:7 with PBS) containing 0.5 mg ml⁻¹ polyethylene glycol (relative molecular mass 20,000) for 1 h at room temperature, followed by further extensive jet-washing in PBS and finally distilled water. The grids were subsequently counterstained with aqueous uranyl acetate for 10 min. Control tests, neither of which gave specific protein A-gold labelling, involved omission of anti-FVIII:Ag antibody and preabsorption of anti-FVIII:Ag antibodies with normal human plasma.



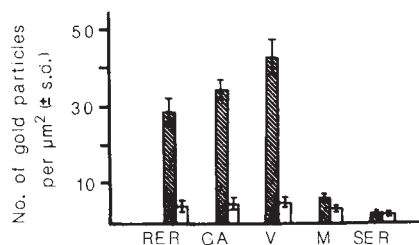


Fig. 2 Intensities of labelling of different compartments of the hepatocytes. ▨, Monoclonal antibody CLB-CAg A followed by protein A-gold complex; □, CLB-CAg A preabsorbed with normal human plasma and followed by protein A-gold. RER, rough endoplasmic reticulum; GA, Golgi apparatus; V, vacuole; M, mitochondria; SER, smooth endoplasmic reticulum.

(Fig. 1b) as well as in vacuoles (Fig. 1c). There were no cases of FVIII:Ag localization limited either to the RER or Golgi apparatus alone, within the same cell. Negligible labelling was found on the membranes of smooth (agranular) endoplasmic reticulum (SER), mitochondria or nuclei. Immunolabelling of sinusoidal endothelial cells is shown in Fig. 1d.

As a specific control for our studies, we performed the following tests. (1) Immunolabelling with CLB-CAg A antibody preabsorbed with a haemophilic plasma; no abrogation of the labelling was observed. (2) Immunolabelling with the same antibody preabsorbed with a normal human plasma. (3) Incubation with protein A-gold complex, alone. In the last two controls, the density of gold particles was very low. Parallel experiments using CLB-CAg A antibody on liver sections of a haemophilic patient did not reveal any positive reaction (Fig. 1e). The results of these experiments are summarized in Fig. 2.

To confirm the validity of our results, a positive control was included. Liver sections were incubated with CLB-RAg35 antibody specific for vWF:Ag¹⁰, then treated with protein A-gold complex; the localization of vWF:Ag in sinusoidal endothelial cells, as demonstrated previously by light and electron microscopy^{13,14}, was confirmed. No vWF:Ag was observed in hepatocytes.

Light microscopic studies^{4,5} using CLB-CAg A antibody have shown the presence of FVIII:Ag in the hepatic sinusoidal endothelial cells, suggesting that these cells are responsible for synthesis of the procogulant antigen^{4,5}. However, the results of biochemical studies suggested that the major site of synthesis of factor VIII procoagulant protein is within hepatocytes⁷.

As we have observed the specific labelling of FVIII:Ag in the rough endoplasmic reticulum of hepatocytes, where protein synthesis is known to occur, we suggest that hepatocytes are largely responsible for FVIII:Ag synthesis. After synthesis (Fig. 1a), FVIII:Ag is transferred from the RER to the Golgi apparatus, where is possibly glycosylated⁸, then transported to the secretory vacuoles (Fig. 1c) and finally discharged, by exocytosis, into the sinusoidal lumen and/or up taken by endothelial cells where it may be stored.

Immuno-ultrastructural studies have been used previously to localize factor VIII-related antigen^{13,14} but not, to our knowledge, factor VIII procoagulant antigen. Here, ultrastructural immunolocalization allowed us to detect FVIII:Ag in the different compartments of hepatocytes and to localize the possible site of FVIII:Ag synthesis. The demonstration of the presence of FVIII:Ag in the rough endoplasmic reticulum of hepatocytes should facilitate further studies on factor VIII procoagulant protein.

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Plakalbumin, α_1 -antitrypsin, antithrombin and the mechanism of inflammatory thrombosis

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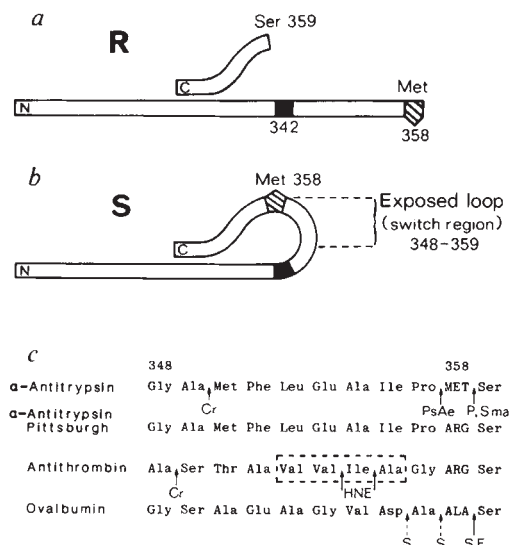
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An old puzzle in protein biochemistry¹ concerns the ready conversion of ovalbumin, by proteolysis, to the much more stable derivative, plakalbumin. Ovalbumin is now known to belong to the serpin superfamily^{2,3}, most of which are serine proteinase inhibitors. We report here studies of two such members of the family, the human plasma proteins α_1 -antitrypsin and antithrombin, and show that they undergo a similar change in stability on selective proteolysis. This change, which is accompanied by a loss of inhibitory activity, can best be considered as an irreversible molecular transition from a native stressed (S) conformation, to a more ordered relaxed (R) form. The maintenance of the native S conformation, and hence the maintenance of inhibitory activity, is critically dependent on the integrity of an exposed loop of polypeptide. We propose that the susceptibility of this peptide loop to proteolytic cleavage gives it an incidental role as a physiological switch which allows the inactivation of individual inhibitors by specific proteolysis. The vulnerability of this exposed loop in each inhibitor also explains the pathological action of a number of venoms and toxins. In particular, the demonstration here of the cleavage of antithrombin, by leukocyte elastase, explains an observed change in blood coagulation that accompanies severe inflammation and which can result in fatal thrombosis.

The proposed S to R conformational change is based on the structure of α_1 -antitrypsin, determined by Löbermann *et al.*⁴, who were able to crystallize the proteolytically cleaved R form of the inhibitor. R α_1 -antitrypsin has a highly ordered structure, and to reconstruct the native S form of the molecule, a strand has to be extracted from the centre of a β -sheet, as shown diagrammatically in Fig. 1. It was predicted that this shift of the strand from a position in which it forms extensive hydrogen bonds to that of the exposed loop in the S structure, would cause a decrease in molecular stability. We have confirmed this change in stability in the experiments detailed in Fig. 2. Human α_1 -antitrypsin was catalytically cleaved at its reactive centre by papain, and human antithrombin was similarly cleaved near its reactive centre by human neutrophil elastase (Fig. 1). The site and completeness of cleavage were confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and automated amino-terminal sequencing⁵. The stability of the native (S) and cleaved (R) inhibitors was determined by heating at a range of temperatures for up to 4 h. Heat-precipitated protein was centrifuged out and the remainder in solution was measured by electroimmunoassay. Over 50% of native antithrombin and α_1 -antitrypsin precipitated after 2 h at 60 °C, but no precipitation

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Fig. 1 *a*, The structure of the serpins is based on R α_1 -antitrypsin which is formed by cleavage at the reactive centre, Met 358. To reconstruct the native S structure (*b*), a strand comprising residues 342–358 has to be removed from the centre of a β -sheet to give an exposed stressed loop. The sequence of this loop (*c*) provides accessible cleavage points for catalytic inactivation of the inhibitors by snake venom (Cr) and bacterial proteases (PsAe, Sma; see below). Antithrombin has a hydrophobic 'switch sequence' (boxed) which is susceptible to catalytic cleavage by human neutrophil elastase allowing physiological inactivation of inhibitory activity. α_1 -Antitrypsin is not subject to catalytic cleavage by elastase even in the Pittsburgh mutant which has no elastase inhibitory activity. P, papain⁵; S, subtilisin⁷; E, pancreatic elastase⁸; HNE, human neutrophil elastase; Cr, proteinase II of *Crotalus adamanteus* venom¹⁷; Sma, metalloproteinase of *Serratia marcescens*²⁰; Psae, *Pseudomonas aeruginosa* elastase¹⁹. The cleavage of antithrombin by neutrophil elastase was confirmed by sequenator analysis which gave equal yields of new N-terminals Ala-Gly-Arg-Ser... and Ile-Ala-Gly-Arg-Ser, continued to 8–12 cycles.



occurred with the cleaved inhibitors in the same conditions. Cleaved α_1 -antitrypsin showed no precipitation until after incubation for 2 h at 80 °C and even at 95 °C some 85% remained in solution after 2 h.

This striking increase in stability following catalytic cleavage was immediately reminiscent of the results of Linderström-Lang and Otteson^{6,7} with ovalbumin, nearly 40 years ago; they observed that subtilisin cleaved ovalbumin at what we now know to be sites homologous to the proposed loop sequence of the other serpins (Fig. 1). The consequent derivative, plakalbumin, had quite different properties; in particular, it had a 16-fold increase in solubility in ammonium sulphate, and formed crystals of different structure from those of ovalbumin. These and subsequent⁸ observations with ovalbumin, together with our own with antithrombin and α_1 -antitrypsin, suggest that an S $\rightarrow$ R transition may be a general means of inactivation of the serpins.

If evolution has conserved this S(active) $\rightarrow$ R(inactive) conformational change in these inhibitors, has it also provided specific cleavage points in the exposed peptide loop to trigger the transition? To test this notion, we examined the inactivation of antithrombin by neutrophil elastase. This choice of inhibitor and enzyme was based on the observation⁹ that blood coagulation is altered in inflammation due to the inactivation of antithrombin by proteases released by neutrophil leukocytes. Isolated human antithrombin was incubated at 37 °C, in the absence of heparin, with human neutrophil elastase, enzyme/inhibitor 1:100(w/w) (see Fig. 2 legend). Sequential samples were removed and the thrombin inhibitory activity determined (Table 1). Complete loss of inhibitory activity occurred within 5 min. Automated amino-terminal sequencing for eight cycles showed that the neutrophil elastase had cleaved the antithrombin at a hydrophobic sequence Val-Val-Ile-Ala in the exposed loop just preceding the reactive centre (Fig. 1). To test whether the corresponding site in α_1 -antitrypsin, Leu-Glu-Ala-Ile, was similarly susceptible to catalytic cleavage by elastase, we used the reactive-centre mutant¹⁰ α_1 -antitrypsin Pittsburgh 358 (Met $\rightarrow$ Arg). This human variant has lost the ability to inhibit porcine pancreatic elastase but is a highly effective inhibitor of thrombin. Table 1 shows the results of incubation of antithrombin and α_1 -antitrypsin Pittsburgh with porcine pancreatic elastase (conditions as described for neutrophil elastase). There was an immediate loss of inhibitory activity of antithrombin but no loss of activity of α_1 -antitrypsin Pittsburgh. As with the other experiments, specificity of cleavage was monitored by SDS-PAGE.

The finding of an elastase cleavage site present in antithrombin but absent from α_1 -antitrypsin, supports the proposal that the exposed loop preceding the reactive centre has been utilized by evolution as a switch to allow specific inactivation. The conclusion that antithrombin is catalytically inactivated by neutrophil elastase agrees with other recent findings^{11,12} of a

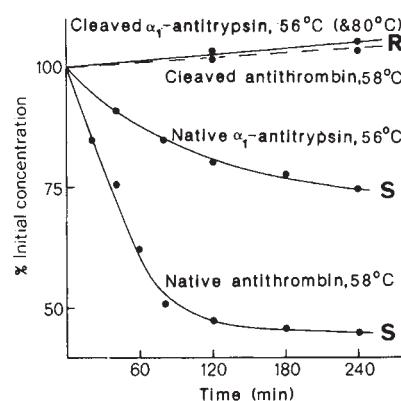


Fig. 2 Solubility of native and specifically cleaved inhibitors on prolonged heating. (The apparent increase in concentration above 100% of the cleaved inhibitors is due to evaporation.) Cleavage: 4 mg of purified α_1 -antitrypsin was dissolved in phosphate-buffered saline pH 6.1 containing 2 mM cysteine; 20 μ l of papain (Sigma P3125, 28 mg ml⁻¹) was added and the mixture was incubated at 37 °C. Samples were taken at 5-min intervals and elastase inhibitory activity assayed using succinyl-(L-alanyl)₃-p-nitroanilide. When 80% had been inactivated the reaction was stopped by adding excess iodoacetamide (5 mg) and the pH was adjusted to 8 with 0.5 M NaHCO₃. SDS-PAGE was performed to determine extent of cleavage near the active site. Purified antithrombin was similarly cleaved with human neutrophil elastase (1:100 enzyme/inhibitor). Antithrombin activity was determined in the presence of heparin by adding excess thrombin and assaying residual thrombin activity with tosyl-Gly-Pro-Arg-p-nitroanilide acetate. The reaction was stopped by adding purified human α_1 -antitrypsin (0.1 mg ml⁻¹). Heat stability: 50 μ l of solution (0.1 mg of inhibitor) was diluted with 450 μ l of 0.75 M Tris-glycine-phosphate buffer (pH 7.5) in a 1.5-ml centrifuge tube, which was placed in a water bath. Samples of 10 μ l were taken at 20-min intervals following centrifugation, and inhibitor concentration determined by electroimmunoassay.

series of control mechanisms that alter proteolysis at an inflammatory site. A central event in inflammation is the release by neutrophil leukocytes of both elastase and oxygen radicals. The oxygen radicals have a limited radius of activity but within that radius they readily oxidize the methionine at the reactive centre of α_1 -antitrypsin, which consequently loses its ability to inhibit elastase. The loss of elastase inhibitory activity by α_1 -antitrypsin allows released neutrophil elastase to break down local connective tissue and to inactivate other inhibitors including antithrombin and C1-inhibitor¹³. This sequence of events,

Table 1 Per cent thrombin inhibitory activity after exposure to protease

Minutes:	0	5	20	40	90
Antithrombin + neutrophil elastase	100%	Nil	—	—	—
Antithrombin + pancreatic elastase	100%	Nil	—	—	—
Antitrypsin Pittsburgh + pancreatic elastase*	100%	96%	99%	100%	88%

* Elastase activity checked at 0 and 90 min.

with an S→R inactivation of inhibitors, provides a molecular explanation for the observed modification of the proteolytic cascades, such as coagulation and the complement system, that occur locally in inflammation.

The localized modification of coagulation is a physiological process but it becomes pathological when massive leukocyte activity occurs, as in septicaemia or endotoxaemia. In these circumstances a significant loss of circulatory antithrombin can occur and life is threatened by intravascular coagulation and thrombus formation⁹. The results reported here provide encouragement for the prospect of treating these diseases. The antithrombin analogue, α_1 -antitrypsin Pittsburgh, is now being produced, using recombinant DNA techniques, by *Escherichia coli* and yeast^{14,15}. Given intravenously, this engineered α_1 -antitrypsin should provide an effective replacement for antithrombin¹⁶ and will have the added advantage over the natural antithrombin that it is not susceptible to an elastase-catalysed S→R inactivation.

The concept we propose, of inactivation of the serpins triggered by proteolysis of an exposed loop, is also in accord with the findings of others^{17–20} to explain the action of a number of venoms, bacterial toxins, and endogenous metalloproteinases²². As illustrated in Fig. 1, these include proteases which give specific cleavage within the loop sequence with consequent inactivation of inhibitors.

The general occurrence of an S→R transformation in the serpins also raises interesting possibilities for the control of other facets of their function. For example, one member of the family is angiotensinogen²¹, which has a key physiological role as the source of the peptides angiotensin I and II which control blood pressure. Angiotensinogen, however, is not, as far as is known, a protease inhibitor—so why retain this complex tertiary structure as a source of a 10-residue amino-terminal peptide? One possibility is that the S→R transformation provides an additional level of control by altering access to, or release of, these terminal peptides.

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Role of the 3' splice site consensus sequence in mammalian pre-mRNA splicing

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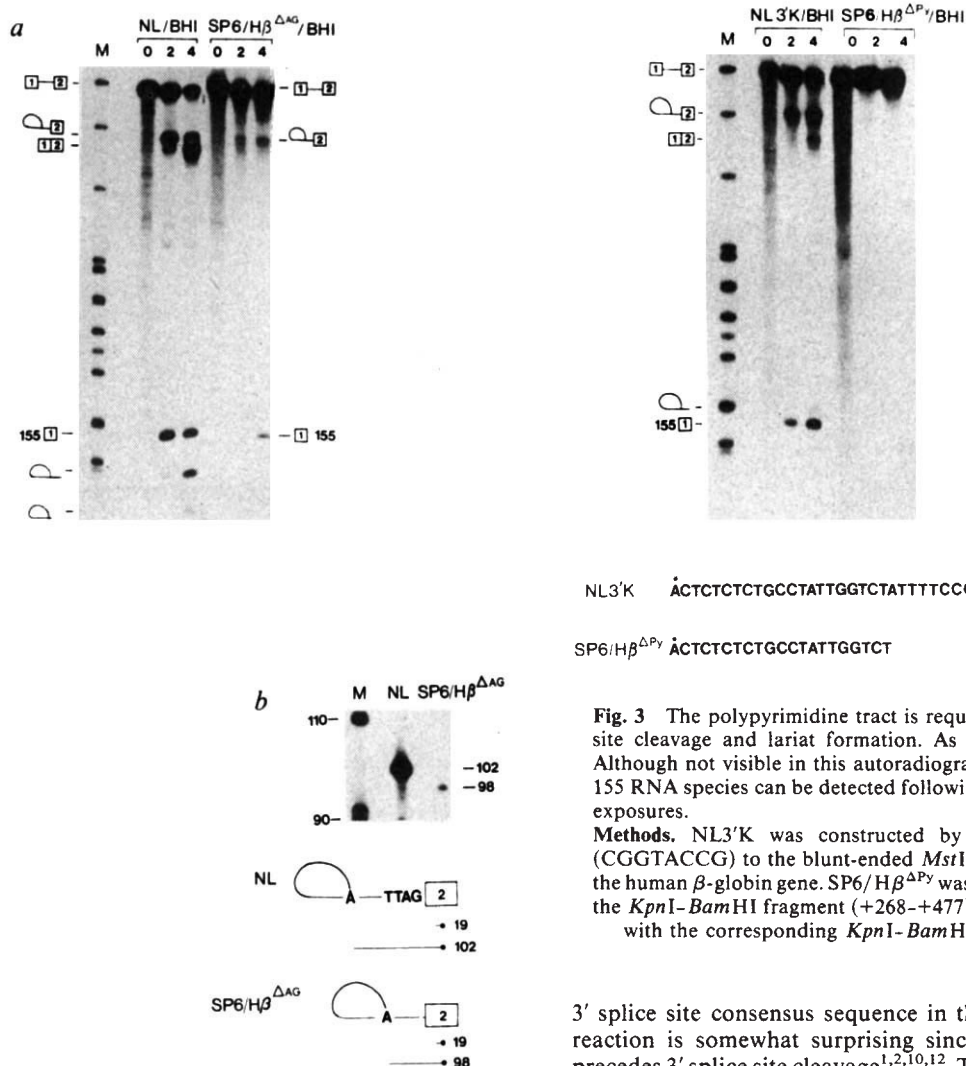
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Pre-mRNA splicing has been shown to occur by a two-step pathway^{1,2}. In the first stage, the pre-mRNA is cleaved at the 5' splice site, generating the first exon RNA species and an RNA species composed of the intron and second exon (IVS1–exon 2 RNA species). In the second stage, cleavage at the 3' splice site and ligation of the exons occurs, resulting in the excision of the intact intron. The excised intron and IVS1–exon 2 RNA species are in the form of a lariat in which the 5' end of the intron is joined to an adenosine residue near the 3' end of the intron by a 2'–5' phosphodiester bond^{1–6}. Here we show that although cleavage at the 3' splice site does not occur until the second stage of the splicing reaction, at least a portion of the 3' splice site consensus sequence is necessary for 5' splice site cleavage and lariat formation. Thus, in higher eukaryotes at least three sequence elements participate in the initiation of the splicing reaction: the 5' splice site, 3' splice site consensus sequence and the RNA branchpoint.

A useful approach for elucidating the mechanism(s) of pre-mRNA splicing has been to analyse the processing of RNA substrates containing naturally occurring or *in vitro*-generated mutations. Mutations in the 3' splice consensus sequence, which includes the polypyrimidine tract and AG dinucleotide at the 3' splice junction⁷, can decrease or eliminate the production of accurately spliced RNA *in vivo*⁸. However, the intermediates and products generated *in vivo* from pre-mRNAs containing mutations within the 3' splice site consensus have not been characterized. Thus, the specific step(s) in the splicing pathway involving the 3' splice site consensus sequence remains unknown. By characterizing the RNA processing products and intermediates generated from mutant RNA substrates *in vitro*, we have found that sequences at the 3' end of IVS1 are required for 5' splice site cleavage and lariat formation. While this work was in preparation a similar conclusion was reached based on the analysis of a β -thalassaemia deletion mutant⁹.

To determine whether the 5' splice site is cleaved in the absence of the branchpoint sequence and/or 3' splice site, the *in vitro* processing of several 'run-off' SP6/ β -globin RNA substrates was examined (Fig. 1a). Accurate cleavage at the 5' splice site generates an RNA species composed solely of the first exon (155 RNA species). The 155 RNA species is generated from the normal (NL) SP6/ β -globin pre-mRNA (NL/BH1, Fig. 1a) and from an SP6/ β -globin transcript that contains exon 1, IVS1, and only 12 nucleotides (nt) of exon 2 (NL/AccI, Fig. 1a). However, when an RNA substrate lacking both the authentic branchpoint sequence and 3' splice site (NL-X/XbaI; Fig. 1a) is processed under the standard *in vitro* splicing conditions^{1,10}, the 155 RNA species cannot be detected. Thus, the presence of an intact, normal 5' splice site is not sufficient for 5' splice site cleavage. To test whether the 5' splice site and branchpoint sequence are together sufficient for 5' splice site cleavage, the LRX2/XbaI RNA substrate, which contains the 5' splice site and authentic branchpoint but completely lacks the 3' splice site consensus sequence, was processed *in vitro*. Again, the 155 RNA species is not detectable (Fig. 1a).

The results obtained using the RNA substrates terminated within IVS1 were confirmed by analysing an RNA substrate derived from a deletion mutant lacking the 16 nt at the 3' end of IVS1 and the first 7 nt of exon 2 (SP6/H $\beta^{\Delta PyAG}$). This RNA substrate also does not undergo detectable 5' splice site cleavage *in vitro* (Fig. 1b). Taken together, these and other results (see below) demonstrate that all or part of the 3' splice site consensus sequence is required for 5' splice site cleavage and lariat formation.



and a diagram of the primer extension products are shown below the autoradiogram. The position of the ³²P-end label is indicated by an asterisk.

Methods. Primer extension analysis was performed as described previously^{1,11}. SP6/Hβ^{ΔAG} was derived from an unpublished linker-replacement mutant (LRX4) in which the sequences CTTAGG at the 3' splice site have been replaced by the *Xba*I linker CTCTAGAG. SP6/Hβ^{ΔAG} was derived by cleavage of LRX4 with *Xba*I followed by treatment with mung-bean nuclease and intra-molecular ligation.

and lariat formation can occur in the complete absence of an AG dinucleotide downstream from the branchpoint.

Finally, we examined the role of the polypyrimidine tract in the 5' splice site cleavage reaction. The construct SP6/Hβ^{ΔPy} contains the splice junction AG dinucleotide and all downstream sequences, but lacks 14 nt of the original 17-nt polypyrimidine tract (Fig. 3). *In vitro* processing of the control RNA substrate (NL3K/BHI) results in efficient 5' splice site cleavage as well as accurate splicing to the AG dinucleotide (Fig. 3 and data not shown). Compared with the NL3K/BHI RNA substrate, the efficiency of 5' splice site cleavage of the SP6/Hβ^{ΔPy}/BHI RNA substrate is drastically reduced (Fig. 3). This result directly demonstrates that the polypyrimidine tract is an important sequence element for 5' splice site cleavage and lariat formation.

The results from these studies indicate that in higher eukaryotes 5' splice site cleavage and lariat formation is dependent upon the 3' splice site consensus sequence. Moreover, the polypyrimidine tract appears to be a more essential component than the AG dinucleotide, although both sequence elements are probably necessary for maximal efficiency. The requirement for the

NL3K $\dot{\text{A}}\text{CTCTCTCTGCCTATTGGTCTATTTTCCACCCCTTGGTACCGTAGGCTGCTGGTG}$

SP6/Hβ^{ΔPy} $\dot{\text{A}}\text{CTCTCTCTGCCTATTGGTCT}$

CGGTACCGTAGGCTGCTGGTG

Fig. 3 The polypyrimidine tract is required for efficient 5' splice site cleavage and lariat formation. As in the legend to Fig. 1. Although not visible in this autoradiogram, small amounts of the 155 RNA species can be detected following long autoradiographic exposures.

Methods. NL3K was constructed by adding a *Kpn*I linker (CGGTACCG) to the blunt-ended *Mst*III site at position +268 of the human β-globin gene. SP6/Hβ^{ΔPy} was constructed by replacing the *Kpn*I-*Bam*HI fragment (+268-+477) of SP6/Hβ^{ΔPyAG} (Fig. 1) with the corresponding *Kpn*I-*Bam*HI fragment of NL3K.

3' splice site consensus sequence in the 5' splice site cleavage reaction is somewhat surprising since 5' splice site cleavage precedes 3' splice site cleavage^{1,2,10,12}. The most likely interpretation of this result is that in higher eukaryotes a factor involved in 5' splice site cleavage and lariat formation associates with the pre-mRNA by recognition of the 3' splice site consensus sequence. In this regard, we have recently shown that a factor(s) associates with the RNA branchpoint early during pre-mRNA splicing. Moreover, this branch point-factor(s) interaction is dependent upon the presence of the 3' splice site consensus sequence¹³. The accompanying manuscript¹⁴ demonstrates that in contrast to higher eukaryotes, in yeast, the 3' splice site consensus sequence is not required for efficient 5' splice site cleavage and lariat formation.

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Cleavage of 5' splice site and lariat formation are independent of 3' splice site in yeast mRNA splicing

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Analysis of messenger RNA splicing in yeast and in metazoa has led to the identification of an RNA molecule in a lariat conformation. This structure has been found as an mRNA splicing intermediate *in vitro*^{1,2} and identical molecules have been identified *in vivo*³⁻⁵. Lariat formation involves cleavage of the precursor at the 5' splice site (5' SS) and the formation of a 2'-5' phosphodiester bond between the guanosine residue at the 5' end of the intron and an adenosine within the intron^{1,2}. The yeast branchpoint is located within the absolutely conserved TACTAAC box (that is, the last A of the TACTAAC box is the site of formation of the 2'-5' phosphodiester bond with the 5' end of the intron)^{3,4}. Moreover, efficient 5' SS cleavage and lariat formation require proper sequences at the 5' splice junction and within the TACTAAC box⁶⁻¹². Here we demonstrate that 5' SS cleavage and lariat formation take place *in vitro* in the absence of the 3' SS and much of the 3' junction. These results are discussed in light of possible differences between yeast and metazoan mRNA splicing.

A pre-mRNA splicing substrate was prepared as described in Fig. 1 legend. To truncate this RNA, segments of the precursor were removed using an RNase H activity and three synthetic DNA oligonucleotides complementary to the fusion transcript (Fig. 1). Conveniently, the source of the RNase H activity was the yeast splicing extract itself^{13,14}, as we discovered that the addition of a complementary oligonucleotide to the precursor RNA and the splicing extract results in ATP-independent site-specific cleavage of the RNA (Fig. 2). Comparison of the RNA fragments generated in the yeast extract with those generated with purified *Escherichia coli* RNase H shows that both enzymatic activities have similar specificities (Fig. 2). The absence of the 3' half of the molecule after incubation in the yeast extract is probably due to the presence of a 5' exonuclease in the yeast extract. Presumably, degradation of the 5' half of the molecule is blocked by the cap structure.

SP6/RP51AΔ2 precursor RNA is efficiently spliced in the yeast extract in the presence of ATP (ref. 15 and Fig. 3a, b). The addition of oligonucleotide RB1 to the precursor RNA and extract reduces the length of the 3' exon from 320 nucleotides (nt) to 10 nt but does not alter the fidelity or extent of the splicing reaction (Fig. 2c, d). The excised intron and 5' exon (whose sizes are independent of the 3' exon) are in similar amounts to when full-length precursor RNA is used as the splicing substrate. The mRNA, 87 nt in length, and the lariat intermediate, which migrates with a relative molecular mass of 192 nt, are likewise present in amounts and proportions similar to the control reaction (Fig. 3b).

Previously published observations have demonstrated the necessity for a functional TACTAAC box to obtain cleavage at the 5' SS^{7,11}. Consistent with these experiments, separation of the 5' exon from the TACTAAC box with the RB27 oligonucleotide results in the formation of an RNA molecule unable to undergo the 5' SS cleavage (Fig. 3e, f). The expected product of this reaction is the 77-nt 5' exon (as in Fig. 3b) and none is visible.

The entire 3' exon and approximately 15-22 nt from the 3' end of the intron are removed by incubation of the SP6/RP51AΔ2 precursor RNA with the RB23 oligonucleotide (Figs 1, 4). With the addition of ATP to this reaction, 5' SS cleavage and lariat formation (150-nt species) occur normally (Fig. 3g, h). Since there is neither a 3' SS nor a 3' exon, the lariat and the 5' exon are the expected terminal products of this

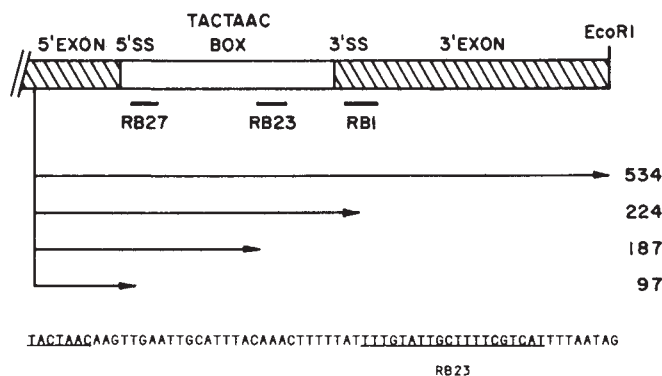


Fig. 1 Pre-mRNA splicing substrate. Hatched lines represent the 5' exon (77 nt) and 3' exon (320 nt) and the open box represents the yeast SP6/rp51AΔ2 intron (137 nt). The positions of hybridization of the synthetic oligonucleotides relative to the 5' splice site (5' SS), 3' splice site (3' SS) and the TACTAAC box are indicated by the heavy lines beneath the gene. The lengths in nucleotides of precursor RNAs, prepared either directly as a runoff product of this gene or by further truncation using the RNase H activity described in the text, are indicated at the right. The sequence of the 3' end of this intron, from the TACTAAC box to the 3' splice site, is given below. The TACTAAC box and the sequence complementary to the RB23 oligonucleotide are underlined.

Method. The construction of plasmid pHZΔ2 and its Sp6 derivative is described elsewhere¹⁵. SP6 polymerase was used to transcribe *in vitro* the intron-containing SP6/RP51AΔ2 fusion gene¹³⁻¹⁵. Transcription of this gene, terminated at the unique *EcoRI* site of the plasmid, generates a 5634-nt run-off product. Although much of the wild-type intron has been deleted from this plasmid, the remaining sequences are sufficient, *in vivo* and *in vitro*, for efficient and accurate splicing^{7,12,15}.

reaction. To demonstrate that the 3' splice site does not participate in an early (before RNase H cleavage) ATP-independent event necessary for lariat formation, the RB23-truncated precursor was gel purified and demonstrated to be an equally good substrate for 5' SS cleavage and lariat formation (data not shown).

To confirm its structure, the putative lariat intermediate from the RB23-truncated precursor RNA was purified and treated with a HeLa cell extract containing a specific 2'-5' phosphodiesterase (debranching enzyme activity)¹⁶. The untreated control has a mobility of approximately 150 nt, while the product of enzymatic debranching has a mobility of 115-122 nt (Fig. 4a, b), the expected length of linear RNA molecules cleaved within the region of complementarity to the RB23 oligonucleotide. The aberrant mobility of this RNA (which is much more dramatic on higher percentage polyacrylamide gels; refs 1, 2 and data not shown) and the effect of debranching, taken together, indicate that the RNA contains a circular component with a 2'-5' phosphodiester bond. Primer extension analysis with the RB1 primer^{7,3} and, most convincingly, direct RNA fingerprint mapping experiments confirmed that the RB23-truncated precursor RNA forms an RNA branch between the proper position of the TACTAAC box and the position 1 G of the 5' SS GTATGT (data not shown), identical to results obtained *in vivo* and *in vitro* and for this and other yeast introns^{3,4,13,15}.

Our results demonstrate that the 3' exon, beyond the first 10 nt, does not provide any detectable sequence or structural information necessary for *in vitro* splicing. While a sequence component would be unlikely, due to the extensive sequence heterogeneity among 3' exons, a minimum length necessary for the splicing apparatus to 'hold' the intron/exon in place could not, *a priori*, be eliminated. Additionally, the separation of the 5' SS from the TACTAAC box results in the formation of a splicing-deficient transcript unable to undergo 5' SS cleavage. This result is in agreement with an earlier observation which demonstrated that, in the absence of a TACTAAC box, full-

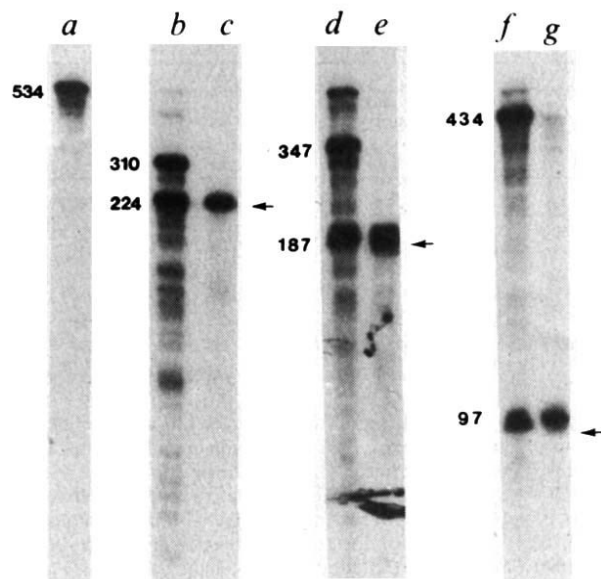


Fig. 2 Comparison of RNA fragments in yeast extract with those generated with *E. coli* RNase H. Lane *a*, undigested precursor DNA. Lanes *b*, *d*, *f*, *E. coli* RNase H digestion of RB1, RB23 and RB27, respectively. Lanes *c*, *e*, *g*, yeast RNase H digestion of RB1, RB23 and RB27, respectively. Sizes (in nucleotides) are indicated on the left. Arrows on the right indicate the products resulting from digestion with the yeast RNase H activity.

Methods. 32 P-labelled RNA was synthesized and gel-purified as described previously¹⁵. RNA was hybridized to synthetic DNA oligonucleotides and digested with *E. coli* RNase H using conditions suggested by the supplier (Bethesda Research Laboratories) or was hybridized with the oligonucleotides in an 8- μ l reaction containing 3.1 mM $MgCl_2$, 3.8% PEG 6000, 75 mM KH_2PO_4 , pH 7.0, and 0.25 μ g of oligonucleotide at 65 °C for 5 min then at 25 °C for 15 min after which 2 μ l of yeast extract (prepared as described in refs 13, 14) was added and the incubation continued for 10 min at 25 °C. All the samples were deproteinized with proteinase K (0.4 μ g ml^{-1} in 100 mM Tris pH 7.5, 2.5 mM EDTA, 150 mM NaCl, 1% SDS), phenol extracted, precipitated with ethanol, and analysed on an 8% 8 M urea acrylamide gel as described elsewhere¹⁵.

length precursor RNA molecules accumulate at the expense of molecules cut at the 5' SS⁷.

Finally, and most interestingly, our results indicate that some and perhaps all of the 3' splice junction sequences play little or no part in the initial events of *in vitro* splicing, that is, intron recognition, 5' SS cleavage and lariat formation. Although the 3' junction, other than the terminal AG, is somewhat ill-defined in yeast⁹, our results show that the removal of approximately 18 (range 15–22) nt of RNA from the 3' end of the intron does not significantly impair 5' SS cleavage and lariat formation. Removal of a comparable number of nucleotides from the 3' SS junction of mammalian introns profoundly inhibits 5' SS cleavage and lariat formation (ref. 17 and accompanying paper¹⁸). While the efficiency of lariat formation is influenced by the sequence context surrounding the TACTAAC box¹², it is likely that the observed spatial arrangement in yeast (that is, the fact that the 3' SS is 8–56 nt downstream of the TACTAAC box⁹) primarily reflects a dependence of 3' cleavage and perhaps exon ligation on the proximity of the branchpoint position. This interpretation is further supported by our observation that the insertion of 78 nt between the TACTAAC box and the 3' SS (at position –25 from the 3' SS) does not markedly inhibit 5' SS cleavage and lariat formation (data not shown).

A comparison of these results with other relevant experiments underscores the notion that substantial differences may exist between yeast and metazoan splicing. In yeast, the RNA branch always forms at the last A of the TACTAAC box^{3,4}. In metazoa, no TACTAAC box exists and the RNA branch forms at more

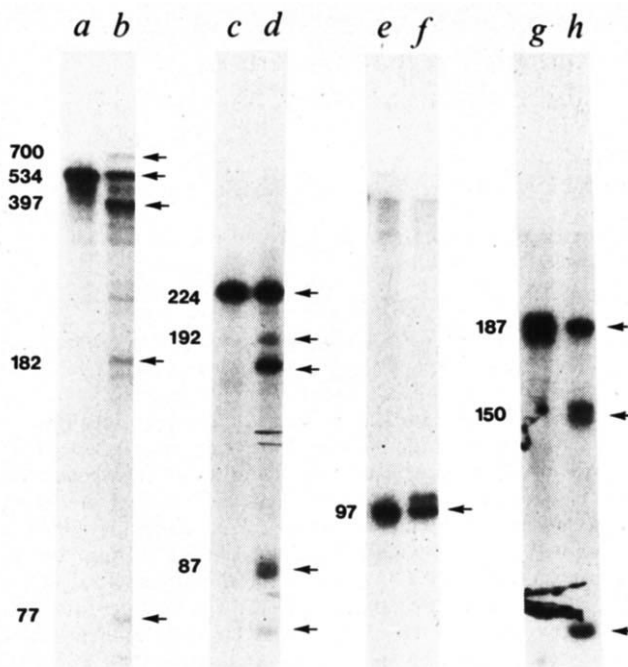


Fig. 3 RNA was prepared as described in Fig. 1. After RNase H digestion, splicing was initiated by the addition of ATP to a final concentration of 4 mM. The splicing reactions were incubated at 25 °C for 10 min, and the RNA analysed as in Fig. 2. Lanes *a*, *c*, *e* and *g* represent full-length precursor RNA and RB1-, RB27- and RB23-truncated precursors, respectively. Adjacent lanes *b*, *d*, *f* and *h* represent the splicing products generated from each precursor after the addition of ATP. Indicated are the measured values, in nucleotides, of the mobilities of the intermediates and products of the splicing reaction relative to denatured DNA standards. The arrows indicate the positions of the relevant RNA species in each reaction. These are, from top to bottom: lane *b*, lariat-intermediate (700 nt), precursor (534), mRNA (397), excised intron (182) and 5' exon (77); lane *d*, RB1-truncated precursor (224), lariat-intermediate (192), excised intron (182), mRNA (87) and 5' exon (77); lane *f*, RB27-truncated precursor (97); lane *h*, RB23-truncated precursor (187), lariat-intermediate (150), and 5' exon (77).

variable sequences^{1,17,19,20}. Indeed, in a HeLa cell extract the branchpoint of SP6/RP51A Δ 2 forms 19 nt downstream of the TACTAAC box¹⁵. These experiments are consistent with the notion that the assignment of the branchpoint position in metazoa is determined in part by sequence (for example, ref. 21) and in part by distance from the 3' SS; recent experiments

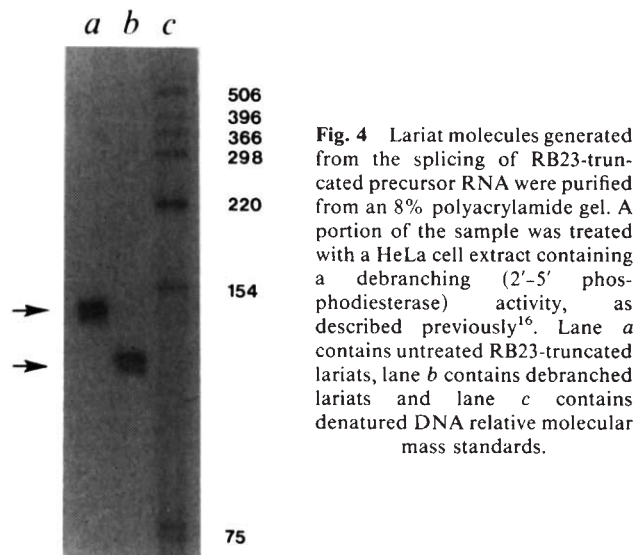


Fig. 4 Lariat molecules generated from the splicing of RB23-truncated precursor RNA were purified from an 8% polyacrylamide gel. A portion of the sample was treated with a HeLa cell extract containing a debranching (2'-5' phosphodiesterase) activity, as described previously¹⁶. Lane *a* contains untreated RB23-truncated lariats, lane *b* contains debranched lariats and lane *c* contains denatured DNA relative molecular mass standards.

suggest that the polypyrimidine stretch is a key portion of the intron for branchpoint placement^{17,18}. All of these considerations predict that in yeast a factor involved in lariat formation (and 5' SS cleavage) may associate with the TACTAAC box independently of recognition of the 3' SS. Recognition of the yeast branchpoint position, that is, the TACTAAC box, may then serve to identify the 3' splice junction (as originally suggested by Langford and Gallwitz⁸).

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Paraneoplastic myasthenic syndrome IgG inhibits $^{45}\text{Ca}^{2+}$ flux in a human small cell carcinoma line

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Certain cancers exert unexplained remote effects on the nervous system¹. Small cell carcinoma (SCC) of the lung, a tumour capable of spike electrogenesis² and which is of possible neural crest origin³, is present in ~70% of patients with the Lambert-Eaton myasthenic syndrome (LEMS)⁴, a disorder characterized by fatigable muscle weakness. Patients with this syndrome have a defect in the (Ca^{2+} -dependent) quantal release of acetylcholine from motor nerve terminals evoked by a nerve impulse or by high K^+ (ref. 5), and a decreased number of presynaptic active zone particles⁶. The physiological and morphological features of the syndrome can be transferred to mice by the patients' IgG, consistent with an autoantibody interfering with the function of voltage-dependent Ca^{2+} channels⁷⁻¹⁰. Here we demonstrate that K^+ -induced $^{45}\text{Ca}^{2+}$ flux in a cultured human SCC line is significantly reduced by LEMS IgG, suggesting that in SCC-LEMS an autoantibody to tumour Ca^{2+} -channel determinants is triggered; its cross-reaction with similar determinants at the motor nerve terminal could lead to the remote neurological syndrome.

Ca^{2+} flux was investigated in the SCC cell line Mar, derived by Dr Morag Ellison (Ludwig Institute for Cancer Research, London) from SCC tumour material obtained from a patient who was not reported to have had neurological symptoms¹¹. This cell line has the same lineage as Mar 27, which shows binding, by immunofluorescence, with UJ13A, a monoclonal antibody specific for determinants of neuroectodermal origin¹².

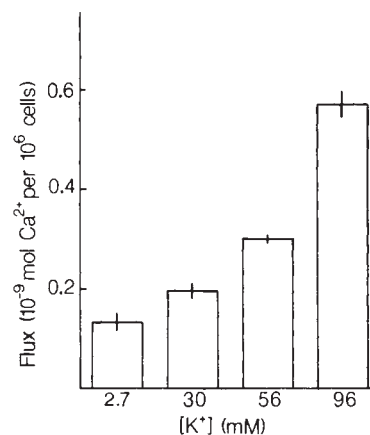


Fig. 1 K^+ -stimulated $^{45}\text{Ca}^{2+}$ flux into human SCC (Mar) cells. Cells were kept in continuous culture in Dulbecco's minimal essential medium with 10% fetal calf serum at 37 °C. The cells grew in non-adherent clusters which were subcultured every 7 days after disaggregation in versene (0.02% EDTA in phosphate-buffered saline). For experimental use, cells were disaggregated using 0.1% trypsin in versene to obtain single-cell suspensions. The reaction was stopped by adding culture medium; cells were then centrifuged (1,100 r.p.m. for 7 min) and resuspended in pre-oxygenated HEPES-Locke buffer (144 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl_2 , 1.8 mM MgCl_2 , 5 mM glucose, 10 mM HEPES, pH 7.2) to give a final cell concentration of $30\text{--}40 \times 10^6$ cells ml^{-1} . Ca^{2+} flux into these cells was measured by a rapid centrifugation method: at time zero, 100- μl aliquots were incubated in tubes containing 250 μl HEPES-Locke buffer, or K^+ -modified HEPES-Locke buffer (KCl substituted for NaCl on an equimolar basis), and 25 μl $^{45}\text{CaCl}_2$ (~225,000 d.p.m.). This gave a final concentration of 10^7 cells ml^{-1} and a final specific activity of ~200 μCi $^{45}\text{Ca}^{2+}$ per mmol Ca^{2+} . At 30 s, triplicate 100- μl aliquots were rapidly transferred to prepared tubes containing 500 μl Ca^{2+} -free HEPES-Locke buffer with additional 1 mM EGTA (to chelate all extracellular calcium) layered above 700 μl of an oil mixture (5:1 Dow-Corning 550/light liquid paraffin). The density of the oil was such that cells could be spun through it to form a pellet. The tubes were centrifuged for 1 min at 11,000g, and flash-frozen in liquid nitrogen. The tip of each tube was cut off, and the pellet solubilized in 0.5% Triton X-100 and counted in an LKB Ultrabeta β -counter using a xylene-based scintillation fluid. Flux at the different K^+ concentrations is expressed as 10^{-9} mol Ca^{2+} per 10^6 cells (mean $\pm$ s.e.m. of triplicate determinations).

The cells have been in continuous culture in our laboratory for 21 months; they grow in clumps typical of SCC cell lines¹³, and contain neurosecretory granules.

The Mar cells exhibited a K^+ -dependent increase in Ca^{2+} flux (Fig. 1) which could be inhibited by D600 (methoxyverapamil) and nifedipine, ligands that block voltage-dependent Ca^{2+} channels¹⁴. Levels of inhibition were similar using either 96 or 56 mM K^+ to depolarize (Fig. 2); Ca^{2+} flux at 2.7 mM K^+ (resting flux) was not inhibitable by these ligands, indicating that flux at this K^+ concentration was not mediated by voltage-dependent Ca^{2+} channels. Resting flux was not inhibited either by the competitive antagonists¹⁵ cobalt (3×10^{-3} M) and manganese (1×10^{-3} M), inhibition being 8% and 0% respectively, or by the Na^+ -channel antagonist tetrodotoxin (3×10^{-5} M; inhibition 0%). By contrast, K^+ -stimulated flux was inhibited both by cobalt (100% at 56 and 96 mM) and manganese (100% at 56 mM and 93% at 96 mM) but not by tetrodotoxin (6% at 56 mM and 0% at 96 mM). The mouse myeloma line P3-NS1-Ag4-1 was studied as a negative control; no stimulated flux was seen at K^+ concentrations up to 96 mM. The resting flux was not inhibited by D600 (data not shown).

These experiments established that the SCC cell line Mar expressed voltage-dependent Ca^{2+} channels comparable in pharmacological characteristics to those studied in the clonal nerve cell line PC12 (refs 14, 15). Similar observations were made in another SCC cell line, UCH-SC1 (derived by Dr Fiona

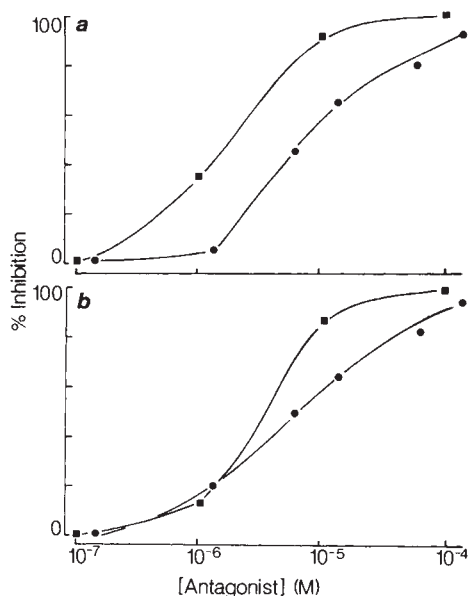


Fig. 2 Inhibitory effect of Ca^{2+} -channel antagonists on K^{+} -stimulated $^{45}\text{Ca}^{2+}$ flux in SCC (Mar) cells. Single-cell suspensions ($30\text{--}40 \times 10^6$ cells ml^{-1}) were incubated with increasing concentrations of nifedipine (■) or D600 (●) at K^{+} concentrations of 56 mM (a) and 96 mM (b) in modified HEPES-Locke buffer (see Fig. 1 legend). The stimulated flux was calculated by subtracting the flux at 2.7 mM K^{+} from that occurring at 56 mM and 96 mM K^{+} , and is expressed as a percentage of the flux obtained in the absence of Ca^{2+} -channel antagonists. Results shown are means of triplicate determinations.

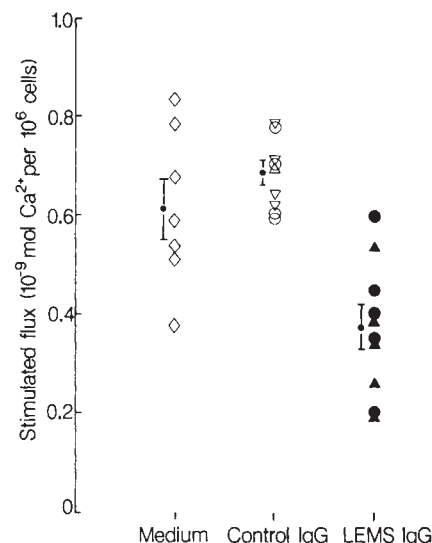


Fig. 3 Effects of LEMS IgG and control IgG on 96 mM K^{+} -stimulated $^{45}\text{Ca}^{2+}$ flux in SCC (Mar) cells. In each experiment, five separate sets of cells were usually assayed: one set cultured in medium alone ($\diamond$), two sets in control IgG and two sets in LEMS IgG. Control IgG was obtained from healthy laboratory workers ($\circ$), neurological controls (see text) (∇), and a SCC patient without LEMS ($\triangle$). LEMS IgG was obtained from patients with associated SCC ($\blacktriangle$) or with no tumour evident ($\bullet$). Stimulated flux (mean of triplicate determinations) was calculated as for Fig. 2.

Moss, University College Hospital, London), that had a similar phenotype to Mar; this finding indicates that voltage-dependent Ca^{2+} channels are probably a feature of SCC cells. Furthermore, physiological studies have shown spike electrogenesis in the SCC cell line DMS 53 which could be blocked by 4 mM MnCl_2 , demonstrating its Ca^{2+} dependence¹⁶.

To investigate the effects of LEMS antibodies on $^{45}\text{Ca}^{2+}$ flux, IgG was prepared as described previously^{7,8} from the plasma of 10 patients with typical clinical and electrophysiological features of LEMS²; five patients had histological evidence of SCC. Control IgG was obtained from the plasma of four healthy laboratory workers, two patients with myasthenia gravis, one patient with the Guillain-Barré syndrome, a patient with chronic progressive inflammatory neuropathy and one patient with SCC without LEMS. Mar cells were cultured for 7 days in the presence of IgG at 4 mg ml^{-1} , a concentration known to be effective *in vivo* for transferring the physiological features of LEMS to mice. Figure 3 shows the stimulated Ca^{2+} flux at 96 mM K^{+} , each point representing one individual; qualitatively similar results were obtained at 56 mM K^{+} (data not shown).

The control IgG-treated cells showed a nonsignificant increase in stimulated flux compared with untreated cells. By contrast, the LEMS IgG-treated cells showed a highly significant decrease in K^{+} -stimulated $^{45}\text{Ca}^{2+}$ flux compared with untreated cells ($P < 0.005$) or with cells grown in control IgG ($P < 0.001$; Student's two-tailed *t*-test). IgG from LEMS patients without evident carcinoma was almost as effective in reducing K^{+} -stimulated Ca^{2+} flux as that from SCC-LEMS patients, the mean values ($\pm$ s.e.) being 0.405 ± 0.065 and $0.346 \pm 0.059 \times 10^{-9}$ mol Ca^{2+} per 10^6 cells respectively. There was no significant difference in resting flux between the groups. Results in a further experiment using a LEMS IgG preparation showed that the inhibition of stimulated flux was proportional to the concentration of IgG in the medium. Thus, whereas IgG at 4 mg ml^{-1} inhibited stimulated flux (96 mM K^{+}) by 68%, concentrations of 0.83 and 0.41 mg ml^{-1} inhibited flux by 56% and 18% respectively.

The interference with K^{+} -stimulated $^{45}\text{Ca}^{2+}$ flux by LEMS IgG indicates an action on voltage-dependent Ca^{2+} channels

and is consistent with the physiological effects of LEMS IgG passively transferred to mice which suggest a 40% decrease in functional Ca^{2+} channels at the nerve terminal⁹. The mode of action of the antibody is uncertain, but direct block of Ca^{2+} channels seems unlikely as the *in vitro* physiological effects of the antibody on quantal release of acetylcholine appear to require more than 2 h of incubation¹⁷.

Susceptibility to the neurological syndrome may be influenced by immune response genes. The frequencies of the major histocompatibility antigen HLA-B8 and of the IgG heavy-chain marker Gm(2) are significantly increased in LEMS¹⁸, but are not reported to be increased in SCC *per se*. The results presented here, together with the observation that removal of the tumour may be followed by recovery of the neurological symptoms², suggest that in SCC-associated LEMS the primary autoantibody response is against voltage-dependent Ca^{2+} channels on SCC cells. The results support the view that immunological responses to surface determinants on tumour cells may underlie other 'remote' neurological syndromes that are associated with cancer. In the LEMS patients without evident tumour, we do not know whether the immune response is raised against an occult SCC or some other stimulus.

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Homologous expressed genes in the human sex chromosome pairing region

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The human sex chromosomes share a pair of homologous genes which independently encode a cell-surface antigen defined by the monoclonal antibody 12E7 (refs 1, 2; see refs 3, 4 for review). The X-located homologue, *MIC2X*, escapes X-inactivation⁵ and the equivalent Y-located locus, *MIC2Y*, was one of the first genes shown to reside on a mammalian Y chromosome². By using a bacterial expression system we have previously cloned a complementary DNA sequence corresponding to a *MIC2* gene and have used this probe to show that the *MIC2X* and *MIC2Y* loci are closely related, if not identical⁶. Here we report the use of the cloned probe to confirm the localization of the *MIC2X* locus to the region Xpter-Xp22.32 (ref. 7) and demonstrate, for the first time, that the *MIC2Y* locus is located on the short arm of the Y chromosome in the distal region Ypter-Yp11.2. The *MIC2* sequences and the sequences described in the accompanying papers by Cooke *et al.*⁸ and Simmler *et al.*⁹ are the first which have been shown to be shared by the sex chromosomes in the pairing region.

The Y chromosome plays a pivotal role in mammalian sex determination: its presence is associated with the male phenotype¹⁰ and its absence results in a female phenotype¹¹. This chromosomal sex determination requires that the X and Y

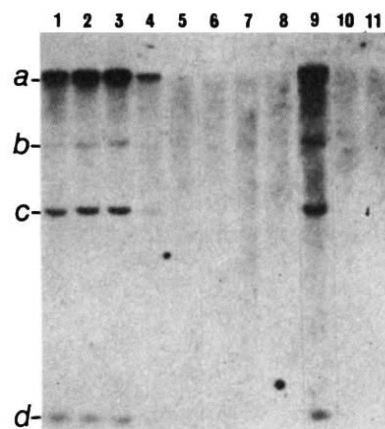


Fig. 1 Chromosomal localization of genomic sequences reacting with pSG1. Lane 1, MOLT-4 (human, XY); 2, GM1416B (human, XXXX); 3, OXEN (human XYYYY); 4, HORL9X (human-mouse hybrid, 'X only'); 5, 1W1-5 (human-mouse hybrid, 'Xq only'); 6, 445x393 (human-mouse hybrid, X-Y translocation + autosomes); 7, AMIR2N (human-mouse hybrid, X-Y translocation + autosomes); 8, UCLAB2 (human-mouse hybrid, deletion Xpter-Xp22.32, + autosomes); 9, 3E7 (human-mouse hybrid, 'Y only'); 10, RAG (mouse); 11, IR (mouse). Four bands (a-d) are visible on the original autoradiogram in all positive lanes (1-4 and 9): a, c and d, human-specific; b present in some mouse cell lines. Relative molecular mass (calculated using *Hind*III-cut $\phi\lambda$ standards, average of three determinations): a, 15.9 kb; b, 5.8 kb; c, 2.9 kb; d, 0.61 kb. Occasionally a weak band at 3.7 kb is also seen. **Methods.** Isolation and characterization of the pSG1 probe will be described in detail elsewhere⁶. Briefly, the cDNA sequence was isolated by screening a $\phi\lambda$ gt11 expression library with the antibody 12E7. A 1-kilobase (kb) *Eco*RI insert from a positive clone was isolated and subcloned into the vector pUC8. This clone was named pSG1. The insert from pSG1 was isolated, nick-translated to a specific activity of $\sim 2 \times 10^8$ d.p.m. μg^{-1} and used as a probe on a Southern blot. DNA preparation, cleavage with *Eco*RI, fragment separation on a 0.8% agarose gel and transfer to a nitrocellulose filter was performed using standard techniques³². The blot was hybridized at 42 °C for 16 h in the presence of $5 \times \text{SSC}$, 40% formamide and 6% polyethylene glycol and washed to a final stringency of $0.1 \times \text{SSC}$ at 65 °C. The filter was exposed to Kodak XAR 5 film for 7 days at -70 °C (see Table 1 for references and details of cell lines.)

Table 1 Comparison of 12E7 antigen expression and reactivity with pSG1

Human-mouse hybrid	Human autosomes*	Human sex chromosomes*	Reaction with pSG1 (see Fig. 1)	Cell surface 12E7 antigen expression†	
				c.p.m. bound × 10 ⁻³ (s.d.) 12E7	p3X63Ag8
Experiments					
HORL9X (ref. 1)	None	X	+	24.6(0.9)	0.9(0.3)
3E7 (ref. 25)	None	Y	+	34.6(1.3)	0.6(0.1)
1W1-5 (ref. 26)	None	Xcen-Xqter	—	0.8(0.1)	0.6(0.1)
445X393 (ref. 2)	2, 3, 4, 6, 7, 11, 14, 18, 19, 21	Yqter-Yq11; Xp22.3-Xqter	—	0.4(0.1)	0.4(0.1)
AMIR2N (ref. 2)	2, 3, 4, 5, 11, 13, 14, 15, 17, 19, 20, 22	Yqter-Yq21; Xp22.3-Xqter	—	0.9(0.2)	0.9(0.2)
UCLAB2 (ref. 7)	1, 2, 3, 7, 11, 15, 17, 19, 20	Xp22.32-Xqter	—	1.5(0.1)	0.9(0.1)
Controls					
OXEN (ref. 27)	Human cell line	XYYYY	+	39.5(2.4)	1.4(0.4)
GM1416B	Human cell line	XXXX	+	29.7(2.3)	0.9(0.1)
MOLT4 (ref. 28)	Human cell line	XY	+	32.2(0.8)	0.3(0.1)
RAG (ref. 29)	Mouse cell line	—	—	0.8(0.1)	0.8(0.1)
1R (ref. 30)	Mouse cell line	—	—	1.8(0.1)	1.4(0.1)

Cell line GM1416B was purchased from the Institute for Medical Research, Camden, New Jersey.

* As determined from a combination of karyotypic and isozyme assays.

† As determined by indirect radioimmunoassay (see refs 1, 2 for details). P3.X63.AG8 is a class-matched negative-control antibody³¹; a positive reaction is arbitrarily regarded as >3 times the control value. Not all the assays were performed at the same time. s.d., Standard deviation.

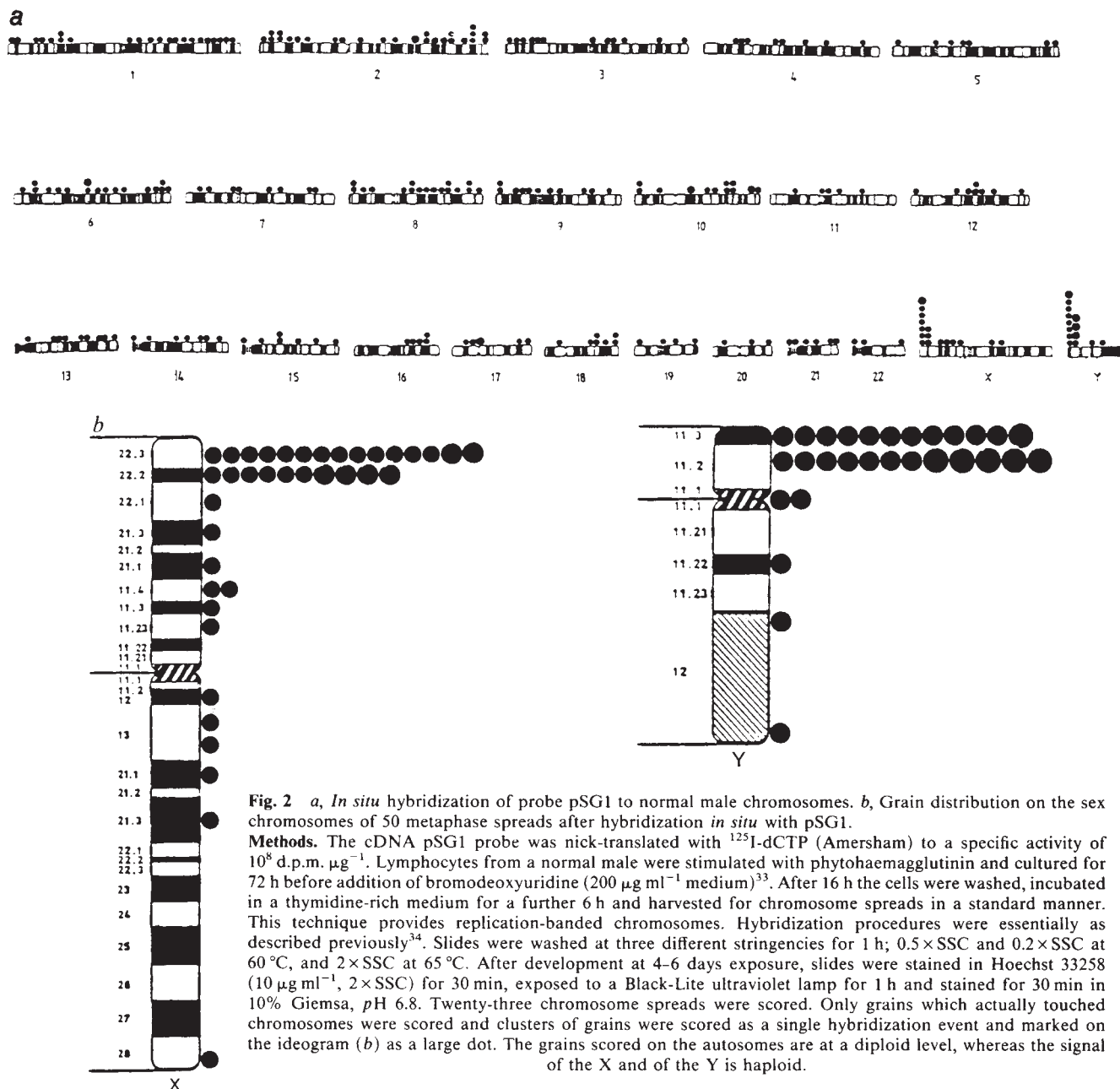


Fig. 2 *a*, *In situ* hybridization of probe pSG1 to normal male chromosomes. *b*, Grain distribution on the sex chromosomes of 50 metaphase spreads after hybridization *in situ* with pSG1.

Methods. The cDNA pSG1 probe was nick-translated with ^{125}I -dCTP (Amersham) to a specific activity of 10^8 d.p.m. μg^{-1} . Lymphocytes from a normal male were stimulated with phytohaemagglutinin and cultured for 72 h before addition of bromodeoxyuridine ($200 \mu\text{g ml}^{-1}$ medium)³³. After 16 h the cells were washed, incubated in a thymidine-rich medium for a further 6 h and harvested for chromosome spreads in a standard manner. This technique provides replication-banded chromosomes. Hybridization procedures were essentially as described previously³⁴. Slides were washed at three different stringencies for 1 h; $0.5\times\text{SSC}$ and $0.2\times\text{SSC}$ at 60°C , and $2\times\text{SSC}$ at 65°C . After development at 4–6 days exposure, slides were stained in Hoechst 33258 ($10 \mu\text{g ml}^{-1}$, $2\times\text{SSC}$) for 30 min, exposed to a Black-Lite ultraviolet lamp for 1 h and stained for 30 min in 10% Giemsa, pH 6.8. Twenty-three chromosome spreads were scored. Only grains which actually touched chromosomes were scored and clusters of grains were scored as a single hybridization event and marked on the ideogram (*b*) as a large dot. The grains scored on the autosomes are at a diploid level, whereas the signal of the X and of the Y is haploid.

chromosomes are incorporated into different germ cells during male meiosis. The human sex chromosomes differ extensively in morphology and in genetic content¹², but meiotic pairing and segregation has been taken to indicate that the sex chromosomes still share sequences¹³. This postulated sequence homology should reside in the morphological pairing region, which occurs between the tip of the X chromosome short arm and the Y chromosome short arm^{14–16}. It has also been suggested that recombination occurs in the pairing region^{13,17} and evidence for this hypothesis has been obtained in the mouse^{18–20}. Despite the apparently compelling arguments for sequence homology in the pairing region, molecular analysis of the human X and Y chromosomes has failed to demonstrate this homology²¹, although extensive homologies were found outside the pairing region^{22–24}.

In a preliminary experiment pSG1 was hybridized to DNA samples prepared from different somatic cell hybrids which had retained either complete human sex chromosomes or defined fragments of human sex chromosomes. At high stringency on Southern blots, pSG1 hybridized strongly to human DNA and only weakly, or not at all, to mouse DNA. The probe also reacted strongly with hybrids which retained complete copies of the

human X or Y chromosome (Fig. 1, lanes 4, 9). Hybrids which retained X chromosomes with terminal short arm deletions failed to react with pSG1: the hybrid UCLAB2 (ref. 7) was particularly informative; this hybrid lacks the human-specific bands and is deleted for the terminal 2 or 3% of the X chromosome, Xpter–Xp22.32. The *MIC2Y* sequences could only be localized to the euchromatic part of the Y chromosome, Ypter–Yq11 (Fig. 1, lanes 7, 9). In these conditions of high stringency no autosomal sequences were detected which cross-reacted with pSG1 (data not shown). In all cases complete concordance was seen between cell surface 12E7 antigen expression on the hybrid and reactivity with the pSG1 probe in the Southern blots (Table 1). The results confirm the previous conclusions which relied solely on expression of the 12E7 antigen^{1,2,7}.

To localize the genomic *MIC2Y* sequence and to confirm the assignment of *MIC2X*, the pSG1 probe was hybridized *in situ* to replication-banded metaphase chromosome spreads derived from lymphocytes of a normal male. Analysis of the silver-grain distribution in 25 cells revealed two major areas of probe hybridization, Xp22.2–pter and distal Yp11.2–pter (Fig. 2*a*). 10% of all grains were scored in these regions, each comprising $\leq 0.5\%$ of the haploid human genome. Of the 22 grains scored

in these regions, 5 were clusters of more than one silver grain; this was 50% of all clusters scored. Furthermore, 50 cells were scored for labelling of the sex chromosomes only (Fig. 2b). The numbers of grains scored on the distal X and Y short arms were similar. Of 38 grains scored on the X chromosome, 67% lay in the bands Xp22.2-pter. On the Y chromosome, 83% of the 29 grains scored lay between the short arm telomere and the distal end of band Yp11.2.

The localization of *MIC2* sequences to the distal short arm of the Y chromosome and the tip of the X chromosome is the first formal demonstration of sequence homology between expressed loci in the predicted pairing regions. Similar homology has been obtained by Cooke *et al.*⁸ and Simmler *et al.*⁹ studying random sequences isolated from Y chromosome cosmid libraries. Evidence has been presented that these anonymous sequences undergo meiotic exchange between the X and Y chromosomes. Based on the sequence homology between *MIC2X* and *MIC2Y*⁶ and the chromosomal localizations described here, we would predict a similar exchange of the *MIC2* loci.

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Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate

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The introduction and expression of foreign genes in plants¹⁻³ will advance our understanding of plant molecular biology and should allow the introduction of new agronomically valuable traits into crop plants. The source of potentially useful genes is large (any living organism may be a donor), but identification of these genes remains difficult. We present here an example of how a gene for herbicide tolerance, an agronomically important trait, can be found in a bacterium and introduced into plants. The herbicide glyphosate inhibits a metabolic step in the biosynthesis of aromatic compounds and the *aroA* gene encodes the inhibited enzyme in bacteria. Therefore, we introduced a mutant allele of this gene that encodes an enzyme less sensitive to glyphosate into tobacco plants. Expression of this gene enhanced tolerance to glyphosate in transformed plants.

N-phosphonomethylglycine (glyphosate) is a widely used broad-spectrum herbicide that kills both weed and crop species. The cellular target of glyphosate in plants is probably the enzyme 3-phosphoshikimate 1-carboxyvinyltransferase (also known as 5-enolpyruvylshikimate 3-phosphate synthase, EC 2.5.1.19; EPSP synthase), which catalyses the formation of 5-enolpyruvylshikimate 3-phosphate from phosphoenolpyruvate and shikimate 3-phosphate⁴. Inhibition of this step of the shikimate pathway causes starvation of aromatic amino acids, accumulation of shikimate^{5,6} and, eventually, cellular death. Tolerance to this compound can be mediated by either overproduction of the target enzyme^{7,8} or by the presence of an altered enzyme⁹. The objective of our present work was to test whether expression in plants of a gene encoding a glyphosate-resistant EPSP synthase confers tolerance to glyphosate. The gene chosen was a mutant allele of the *aroA* locus of *Salmonella typhimurium*⁹

encoding an EPSP synthase in which amino-acid substitution of a proline to serine caused a decreased affinity for glyphosate¹⁰ without affecting the kinetic efficiency of the enzyme (G.T., unpublished observations). We used a T-DNA-based vector to express the gene in tobacco. T-DNA is a region of large plasmids, termed Ti (tumour-inducing) or Ri (root inducing) plasmids, that is transmitted to plant cells on infection by *Agrobacterium tumefaciens* and is stably integrated into the plant genome. This element carries genes that are expressed in the plant cell¹¹. The *aroA* gene of *Salmonella* has been isolated and sequenced¹⁰. To express it in plants we used two different promoters. In the first construct the *aroA* gene was fused to the transcriptional signals from the octopine synthetase gene (*ocs*)¹². A second hybrid gene, *mas-aroA*, was constructed by fusing the mannopine synthase gene (*mas*) promoter^{13,14} and the *tml*^{15,16} polyadenylation signal to the *aroA* gene. These hybrid genes, *ocs-aroA* and *mas-aroA*, are described in Fig. 1. These constructs were cloned

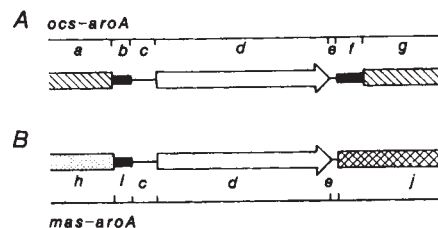


Fig. 1 Detailed structure of the *ocs-aroA* (A) and *mas-aroA* (B) genes. a, *ocs* 5', including 16 base pairs (bp) of transcribed region ending with nucleotide 13,643 (ref. 16); b, linker region, sequence GGAATCCCCGG-ATCCC; c, 5'-untranslated region of *aroA*¹⁰, sequence GTTCTGTTTTT-GAGAGTTGAGTTTC; d, *aroA* coding region, 1,274 bp long¹⁰; e, 3'-untranslated region of *aroA*, 17 bp, to a naturally occurring *Sall* site¹⁰; f, fragment of the tetracycline-resistance gene of pACYC184 (ref. 31), ~150 bp¹⁰ plus a portion of the pUC7 polylinker; g, *ocs* 3' starting at nucleotide 12,823 (ref. 16) and including the 3' portion of the *ocs* coding region (the *ocs* polyadenylation signal is 447 bp from the start of this region); h, *mas* 5', including ~65 bp of transcribed region ending at nucleotide 20,128 (ref. 16); i, linker region, sequence GACTCTAGAGGATCCC; j, *tml* 3' is joined to *aroA* by a *Sall*/*Xho*I hybrid site. An *Xho*I linker was spliced to the DNA termini produced by *Sma*I digestion at nucleotide 11,210 (ref. 16) of *tml* 3'; this region does not include any translated sequence and a transcription polyadenylation signal is 192 bp away. Thus, the transcribed region of the *ocs-aroA* gene is slightly longer than 1,900 bp, whereas that of the *mas-aroA* gene is slightly less than 1,600 bp long.

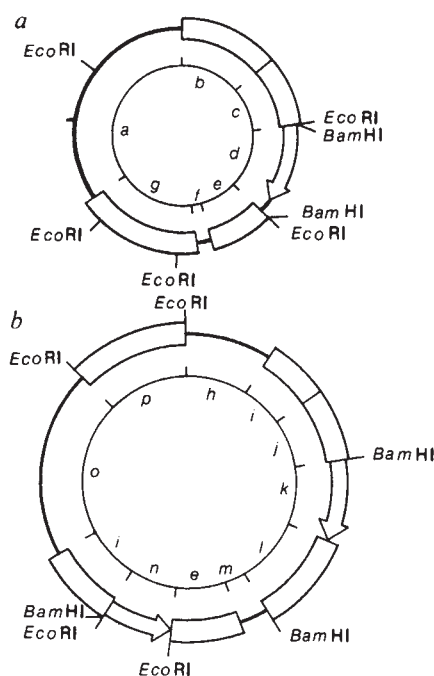


Fig. 2 Genetic map of plasmids pPMG55 (a) and pPMG85 (b). The lettered circle inside each plasmid defines its constituent elements. a, pACYC184 (*Bam*HI to *Hind*III); b, bacterial kanamycin-resistance gene from *Tn5* (ref. 32); c, *ocs* 5', 15,208 to 13,643 (ref. 16); d, *aroA*; e, *ocs* 3', 12,823 to 11,207 (ref. 16); f, *Xho*I to *Bam*HI fragment from the 3' region of the kanamycin-resistance gene of *Tn5*; g, 2.4-kb *Bgl*III fragment from *Hind*III-17 (ref. 33) of pRiA4; h, *Eco*RI to *Hind*III 1.5-kb fragment from pACYC184; i, bacterial kanamycin-resistance gene from pUC4K; j, *mas* 5', 21,476 to 20,128 (ref. 16); k, *aroA*, *Bam*HI to *Sal*I from pPMG34; l, *tml* 3', 11,207 to 9,062; m, *Bam*HI to *Sal*I fragment from the tetracycline-resistance gene of pACYC184; n, *npt* from pCGN552; o, part of pACYC184 from the *Sal*I site to the *Eco*RI site, length 2.5 kb; p, *Eco*RI 2-kb fragment internal to g.

Methods. The plasmids were used to introduce the *aroA* chimaeric genes into the T-L DNA of *Agrobacterium rhizogenes*. To construct pPMG55, pPMG34 (ref. 10) was cut with *Bam*HI and the *aroA* gene in a *Bam*HI fragment was cloned into pUC7 (ref. 34) to give pPMG38. The *aroA* gene was then excised as an *Eco*RI fragment and cloned in pCGN451 (Knauf *et al.*, manuscript submitted). Plasmid pCGN451 is an expression vector consisting of pUC9 and the octopine synthase gene (*ocs*) 5' and 3' regions enclosing a unique *Eco*RI site. Cloning of a gene in such a site allows its transcription in plants and, if properly oriented and arranged¹⁻³, correct translation. Further, the expression cassette, encompassing *ocs* 5', any gene that does not have *Xho*I sites, and the *ocs* 3', can be excised by *Xho*I digestion. Accordingly, the *ocs-aroA* gene was excised by *Xho*I digestion and cloned into pCGN529 (Knauf, unpublished results) resulting in pPMG55. The structure of pCGN529 can be deduced by deleting the *ocs-aroA* gene from pPMG55. It is based on pACYC184 and carries a bacterial kanamycin-resistance gene for selection in *Agrobacterium* and a fragment of the T-L DNA of pRiA4 to allow homologous recombination with the latter. To construct pPMG85 we cloned the mannopine synthetase gene (*mas*) 5' region¹³ from pTiA6. A cosmid clone carrying the T-DNAs of pTiA6, called pVCK232 (ref. 35), was digested with *Eco*RI and the fragment called *Eco*13 or *EcoC* was cloned in pACYC184, giving pCGN14. Digestion of pCGN14 with *Cl*aI and *Sph*I yielded, among others, a fragment from *Cl*aI site 20,128 (ref. 16) to *Sph*I site 21,562 which contains the *mas* 5' and was cloned in the *Sph*I and *Acc*I sites of pUC19 (ref. 36) resulting in pCGN40. The *aroA Bam*HI fragment from pPMG34 was cloned in the proper orientation in pCGN40, fusing this gene to the *mas* promoter and resulting in pPMG67. To provide a polyadenylation signal, the *tml* 3' region¹⁵ of pTiA6 was used. A T-DNA *Bam*HI fragment (nucleotides 9,062-13,774; ref. 16) containing such a region was cloned from pVCK232 in pACYC184 in an orientation such that nucleotide 13,774 was proximal to the *Hind*III site of the vector. An *Xho*I linker was added to the *Sma*I site at position 11,207 and the resulting plasmid termed pBamX. Digestion of pPMG67 with *Hind*III and *Sal*I yielded a fragment containing most of the *mas* 5', and the *aroA* gene. Such a fragment was cloned in pBamX digested with *Hind*III and *Xho*I, generating a *mas-aroA-tml* hybrid gene. This plasmid was called pPMG73. To allow efficient selection in *Agrobacterium*, the kanamycin-resistance gene from pUC4K³⁴ was excised with *Sal*I and cloned in a *Xho*I site present in the *aroA*-distal end of the *mas* 5', giving pPMG76. An *Eco*RI fragment from pRiA4 T-L DNA was cloned in the chloramphenicol-resistance gene *Eco*RI site of pPMG76, yielding pPMG82. To allow selection of transformed plants on kanamycin, a *mas-npt* hybrid gene was constructed. A similar construct has been reported elsewhere¹⁴. Our hybrid gene was constructed as follows: the *mas* 5' was excised from pCGN40 (see above) by digestion with *Eco*RV (position 21,552; ref. 16) and *Eco*RI (in the pUC19 polylinker) and cloned in pCGN451 digested with *Sma*I and *Eco*RI. This latter treatment deletes all the *ocs* 5' from pCGN451 and inserts the *mas* 5' in its place. In addition, part of the pUC19 polylinker, from *Xba*I to *Eco*RI, is placed between *mas* promoter region and the *ocs* polyadenylation site, allowing a choice of different sites for insertion of genes to be expressed. In the *Eco*RI site of this plasmid (termed pCGN46), we inserted an *Eco*RI fragment from pCGN522 (Knauf *et al.*, manuscript submitted) carrying the *Tn5 npt*³² gene from which an untranslated ATG sequence had been removed to optimize translation efficiency. The hybrid *mas-npt-ocs* gene was excised by digestion with *Xho*I and cloned in the *Sal*I site of pPMG82, yielding pPMG85.

into intermediary vectors, yielding plasmids pPMG55 and pPMG85, respectively (Fig. 2). The latter also contains a *mas-npt* hybrid gene (*npt*, neomycin phosphotransferase) that allows selection of transformed plants by growth on kanamycin¹⁴. To allow insertion of *ocs-aroA* and *mas-aroA* into the plant genome, pPMG55 and pPMG85 were incorporated into the T-L DNA of pRiA4 (ref. 17). Co-integrate plasmids generated by recombination of pPMG55 and pPMG85 with pRiA4 were obtained as described previously¹⁸. One of these co-integrate plasmids contained ~10 copies of pPMG55 tandemly arranged in the T-L DNA. Another co-integrate contained a single copy of pPMG85. The strains harbouring these plasmids (termed A4T-55 and A4T-85, respectively), were chosen for plant transformation (Fig. 3).

Tobacco leaf disks were co-cultivated with A4T, A4T-55 and A4T-85 and plants were obtained by the protocol described in Fig. 5 legend. Transformed plants were identified by the production of agropine¹⁹ in the case of A4T and A4T-55 and by selection on kanamycin-containing medium in the case of A4T-85. Two regenerants from each strain co-cultivation were clonally propagated and characterized further; those from A4T-55 were designated 1-1 and 1-7, those from A4T-85 2-1 and 2-4. All appeared to be normal with the exception of 1-7, which had wrinkled leaves and a slower growth habit. DNA was prepared²⁰ from the leaves, digested with restriction endonuclease *Bam*HI or *Eco*RI and subjected to Southern blot analysis with an *aroA*-specific RNA probe. DNA from 1-1 and 1-7 contained the expected 1.5-kilobase (kb) *Eco*RI fragment homologous with *aroA*, and DNA from 2-1 and 2-4 contained the expected 3.5-kb *Bam*HI fragment hybridizing to the probe (Fig. 4). By comparison with a concentration standard, we could approximate the copy number of hybrid *aroA* genes in each transformant: 1-1 plants contained a single copy of the *ocs-aroA* gene per cell; 1-7, 2-1 and 2-4 contained probably 2-4 copies. We detected no hybridization of these genes to DNA of control plants.

Northern blot analysis of polyadenylated RNA^{21,22} isolated from leaves revealed a positive signal corresponding to an RNA species of ~2,000 bases (Fig. 4) in 1-1 and 1-7; this is the expected size of the *ocs-aroA* transcript. The estimated abundance of this RNA was between 0.002 and 0.005% of the total polyadenylated RNA. Similar levels (0.005%) were observed by analysing the expression of *ocs-aroA*, or of the octopine

synthase gene in galls of tomato and turnip plants²³. A slightly shorter messenger RNA of 1,600 bases was found in leaves of 2-1 and 2-4 plants; this corresponds to the expected size of the *mas-aroA* mRNA. The abundance of this mRNA was not estimated.

To test whether these mRNAs were properly translated, we performed Western blot analysis of leaf proteins²⁴. Both *ocs-aroA*- and *mas-aroA*-transformed plants produced a polypeptide reactive with anti-EPSP synthase serum and of identical mobility to pure EPSP synthase. This polypeptide was not found in control plants. The abundance of bacterial EPSP synthase in tobacco leaves varied according to the transformant tested. 1-1 was definitely positive but expressed much less bacterial EPSP synthase than 1-7, 2-1 and 2-4. To determine whether the protein products of the *ocs-aroA* and *mas-aroA* genes were enzymati-

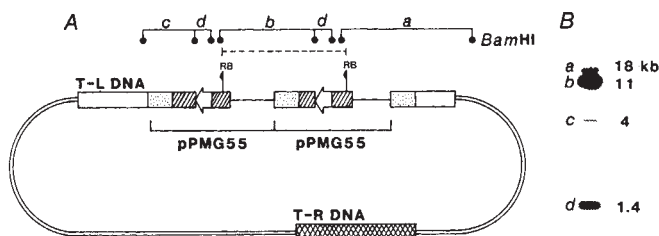


Fig. 3 A, Structure of pRiA4-55. This plasmid was generated by *in vivo* co-integration of pPMG55 and pRiA4 (ref. 18). Multiple recombination events, or the integration of a multimeric form of pPMG55, lead to the presence of ~10 copies of pPMG55 in pRiA4-55. For simplicity, only two pPMG55 moieties are shown. T-L DNA sequences are shown as open boxes, except for the segment that provides homology between pRiA4 and pPMG55, which is dotted. The *ocs* 5' and 3' regions are hatched; *aroA* is represented by the open arrow. The single line represents plasmid pACYC184 and the bacterial kanamycin-resistance gene from Tn5. The T-R DNA area is cross-hatched, while the rest of pRiA4 is shown as a double line. RB designates the right border of pTiA6 T-DNA. This border is part of the *ocs* 5' region and is therefore reiterated with pPMG55. Extensive Southern analysis of regenerated plants suggests that, at least in 1-1 regenerants, the transfer of the *ocs-aroA* into the plant genome occurred by integration of a single copy of pPMG55, probably from right border to right border. The putative region integrated into the genome is marked by a broken line. Plasmid pRiA4T-85 is not shown. It resembles pRiA4T-55 except that only one moiety of pPMG55 is integrated; no border sequences are present in pPMG55; and the region of homology between pRiA4 and pPMG55 is slightly smaller than the one used in pPMG55 (see Fig. 2). B, Southern blot analysis of pRiA4-55 digested with *Bam*HI and probed with pPMG45. The hybridizing bands *a-d* correspond to the segments in A. Band *d* corresponds to the bacterial *aroA* gene (see Fig. 2), and its presence is not affected by the number of pPMG55 moieties integrated in pRiA4. Band *b* is only present when at least two copies of pPMG55 have integrated in a tandem fashion. Bands *a* and *c* are boundary fragments generated by the right and left junctions between pPMG55 and T-L-DNA. These fragments have approximately the same amount of DNA available for hybridization to the probe. Thus, the ratio of intensity between *a+c* and *b+d* is a fair estimate of the number of pPMG55 copies in pRiA4-55.

cally active we used the following strategy: activity of EPSP synthase was measured by the method of Boocock and Coggins²⁵. It was observed that antiserum raised against bacterial EPSP synthase completely inhibited bacterial enzyme activity while the plant enzyme activity remained unaffected, consistent with the observation that plant EPSP synthase does not react with this antiserum. Selective inactivation is particularly useful in distinguishing between plant and bacterial EPSP synthase activity in cellular extracts of transformed plants. The difference in activity between untreated and antiserum-treated extracts represents activity of the *ocs-aroA* (or *mas-aroA*)-encoded EPSP synthase. In transformed plants the bacterial activity amounted to ~2.0 U (nmol anthranilate/min per g fresh weight) for 1-1; 2.7 U for 1-7; 4.9 U for 2-1; and 3.9 U for 2-4. Total EPSP synthase activity was variable, ranging from 9.0 to 38.2 U, contrary to the *aroA*-derived activity that remains close to the given values over several measurements. Control plants typically have no *aroA*-specific EPSP activity, or at the most, amounts <0.5 U. The results presented here provide strong evidence that both hybrid genes are correctly transcribed and translated in plant cells to produce an active bacterial EPSP synthase. Enzymatic and immunological analysis of *aroA* expression in these transformants indicate that 1-1 produces significantly less bacterial EPSP synthase than 2-1 and 2-4. These measurements were repeated several times and confirmed these results.

To test whether the mutant bacterial *aroA* gene could confer tolerance on plants, tobacco plants expressing the gene were sprayed with glyphosate. As shown in Fig. 5, transformed plants exhibit considerable tolerance to glyphosate. We found that tolerance levels were correlated with the level of expression of the *aroA* gene in the plant, 1-1 being the most sensitive of the transformants and 2-1 and 2-4 being the most tolerant. When plants were sprayed with the equivalent of 0.5 kg hectare⁻¹ of the isopropylamine salt of glyphosate under our growth-

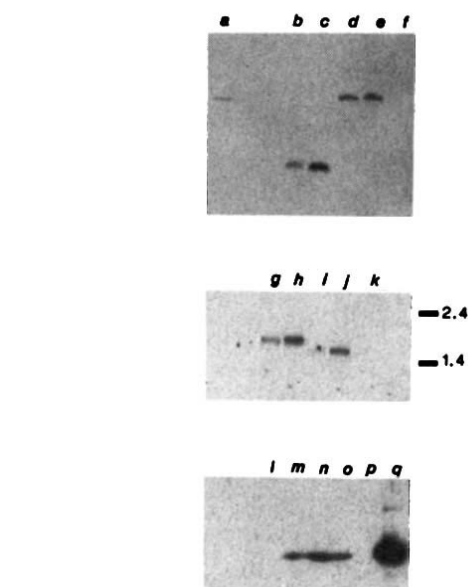


Fig. 4 Detection of the chimaeric *aroA* gene and its expression in transformed plants. *a-f*, Lane *a*, pPMG76 (Fig. 2); *b*, 1-1; *c*, 1-7; *d*, 2-1; *e*, 2-4; *f*, Xanthi nc. (see Fig. 5). DNA was purified from 1 g tissue according to the protocol of Dellaporta²⁰, with the modification that after the first isopropanol precipitation DNA was spooled on a glass rod, resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA and reprecipitated; the spooled DNA was resuspended in water. Aliquots were digested with *Bam*HI (*a, d, e, f*) or *Eco*RI (*b, c*), electrophoresed in 0.7% agarose, denatured and blotted onto nitrocellulose²¹. An RNA probe specific for *aroA* was used. The *Bam*HI to *Sall* fragment of pPMG34 (ref. 10) containing the *aroA* gene was cloned in pSP65 (Promega Biotec, Madison). The constructed plasmid was cut with *Sall* and transcribed *in vitro* with SP6 RNA polymerase (Promega Biotec) in the presence of radioactive GTP. The labelled transcript was used as a probe. Lane *g*, 1-1; *h*, 1-7; *i*, 2-1; *j*, 2-4; *k*, Xanthi nc. RNA was prepared as described²¹, except that 1 mM aurintricarboxylic acid was added to the extraction buffer, fractionated on oligo-dT cellulose (Collaborative Research)²¹, electrophoresed under denaturing conditions through a 1.5% agarose gel and blotted onto a nitrocellulose filter²². Hybridization was as described^{21,22}. The probe used was pPMG38 (see Fig. 2). DNA standards (2.4 and 1.4 kb) were denatured before electrophoresis. *l-q*, Western blot analysis of bacterial EPSP synthase. Lane *l*, 1-1; *m*, 1-7; *n*, 2-1; *o*, 2-4; *p*, Xanthi nc; *q*, 500 ng purified EPSP synthase. About 2.5 g (fresh weight) of leaf tissue was ground in liquid nitrogen, then suspended in 0.1 M Tris, pH 7.5, 10 mM EDTA, 0.15 M NaCl, 0.05% Nonidet P-40, 25 mg ml⁻¹ bovine serum albumin, 1 mM dithiothreitol and 0.13 µg ml⁻¹ leupeptin (Sigma). Samples were homogenized with a Brinkmann Polytron after the addition of 0.5 g polyvinylpyrrolidone, then centrifuged at 15,000g for 15 min at 4 °C. 100 µl of antiserum, prepared by injecting purified EPSP synthase¹⁰ into rabbits, and 250 µl 10% (w/v) suspension of *S. aureus* (Calbiochem) were added to each supernatant and incubated, with agitation, for 16 h at 4 °C. Samples were then centrifuged and the pellet washed twice with 20 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl and 0.05% Nonidet P-40. The resulting pellets were suspended in 100 µl 0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol and heated for 2 min at 90 °C. The entire sample was then electrophoresed on 10% acrylamide gels³⁷. The resolved polypeptides were transferred to nitrocellulose (BA85; Schleicher & Schuell) as described by Burnette²⁴ at 100 V for 2 h in a Hoefer TE42 transfer unit. Nitrocellulose filters were then incubated in BLOTTO³⁸ for 1 h at 42 °C, followed by an overnight incubation at room temperature in BLOTTO containing a 1:50 dilution of anti-EPSP synthase serum. Filters were washed for 10 min in 20 mM Tris, pH 7.5, 150 mM NaCl, for 20 min in the same buffer containing 0.05% Tween-20, and for another 10 min in buffer without detergent. BLOTTO containing 10⁶ c.p.m. ml⁻¹ of ¹²⁵I-labelled protein A (9 µCi mg⁻¹; NEN) was then added to filters and incubated at room temperature for 2 h. The filters were then washed overnight in 50 mM Tris, pH 7.5, 1 M NaCl and 0.4% sarkosyl. After rinsing and drying, filters were exposed to Kodak AR X-ray film at -70 °C using a Dupont Cronex intensifying screen.

chamber conditions (see Fig. 5), *aroA*-negative plants were not killed but after 20 days weighed only 10% of unsprayed controls, whereas transformants 1-1 and 2-4 weighed 30 and 70%, respectively, of the unsprayed controls. Five weeks after the treatment, 2-4 plants weighed more than the unsprayed control because of growth of lateral shoots. Thus, expression of *aroA* in tobacco did not result in complete loss of sensitivity to glyphosate but

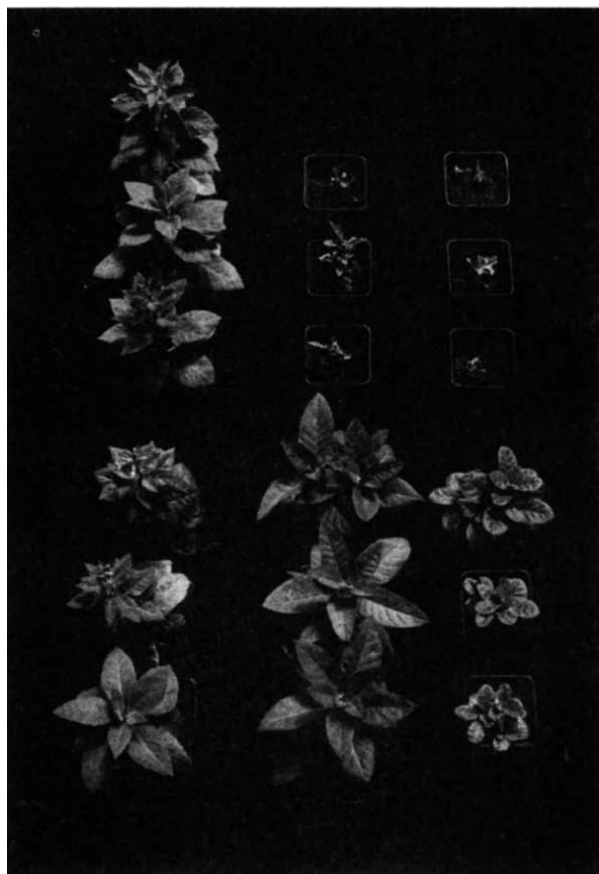


Fig. 5 Phenotype of transformed plants. Regeneration of *A. rhizogenes*-transformed plants has been accomplished previously^{39,40}. Transformed plants were generated as follows. *Nicotiana tabacum* cv. 'Xanthi nc' was grown under axenic conditions⁴¹. Disks 2 mm in diameter were punched from young leaves (2 cm long) and incubated in liquid MS medium (1 mg l⁻¹ naphthalene-acetic acid, 1 mg l⁻¹ kinetin) in the dark at 23 °C. *Agrobacterium* strains were grown overnight in MG/L broth⁴² at 30 °C and added to the disks to obtain a final bacterial cell concentration of 5–10 × 10⁶ ml⁻¹. Co-cultivation lasted 24 h under the above conditions. Disks were then transferred to solid MS medium (0.7% Phytagar, Gibco-BRL; 350 mg l⁻¹ cefotaxime, no hormones and in the case of disks co-cultivated with A4T-85, 100 mg l⁻¹ kanamycin) and incubated in the light (50 µE m⁻² s⁻¹, 12 h light per day) at 23 °C. Roots growing from the edges of the disks were excised and placed on solid shooting MS medium (2 mg l⁻¹ indole-3-acetic acid (IAA), 2 mg l⁻¹ kinetin). Shoots were rooted and propagated in MS medium (1 mg l⁻¹ IAA, 0.15 mg l⁻¹ kinetin). Putative A4T-85 transformant shoots were rooted in kanamycin-supplemented medium (100 mg l⁻¹). *ocs-aroA*, *mas-aroA* and control transformed plants were obtained from clonal propagation of individual regenerants. Plants were photographed 40 days after they had been sprayed with a commercial formulation of glyphosate. At the time of spraying plants had on average six leaves, were growing actively and were <10 cm tall. They were grown at 25 °C, 200 µE s⁻¹, 90% relative humidity and 12 h light per day. The application rate of glyphosate was 0.6 and 1.0 kg hectare⁻¹. The glyphosate-treated plants and the respective untreated controls were transferred to a greenhouse 15 days after spraying. Top nine plants, Xanthi nc; bottom nine plants, 2–4; left vertical row, unsprayed controls; middle row, treated with glyphosate at 0.6 kg hectare⁻¹; right row, treated with glyphosate at 1.0 kg hectare⁻¹. Plants transformed by the vector alone responded to glyphosate treatment, similar to Xanthi nc.

in levels of tolerance depending on the expression level. A precise quantitation of these two variables is now underway.

Many sites of action of glyphosate in plants have been proposed²⁶; most evidence suggests that EPSP synthase is the major, if not the sole, target site. First, genetic analysis demonstrated it to be the main site of inhibition in enteric bacteria^{7,9}; second, EPSP synthase is the only enzyme tested that is highly sensitive to glyphosate²⁵; third, plant tissue selected in culture for resistance to glyphosate exhibits severalfold overexpression of EPSP synthase⁸; and fourth, treatment of plants with glyphosate results in accumulation of high levels of the precursor shikimate in the vacuole, presumably from dephosphorylation of phosphoshiki-

mate, a substrate of EPSP synthase⁵. The glyphosate-tolerant phenotype of plants expressing the *aroA* gene is in agreement with the above data. However, we cannot exclude the possibility that other targets exist as the phenotype observed has partial tolerance. Further improvement of bacterial *aroA* expression and targeting of the protein to the chloroplast^{27,28} should achieve higher tolerance levels.

The shikimic acid pathway seems to be located in plastids and possibly in the cytoplasm^{29,30}. EPSP synthase is not a secretory protein in *Salmonella*. We expect it to be localized in the cytoplasm when expressed in plant cells. Failure of an active enzyme to induce a tolerant phenotype may have been interpreted in previous studies as an indication that the cytoplasmic pathway of shikimate biosynthesis is either non-existent or of little consequence to the total cellular biosynthesis of chorismate. On the other hand, the induction of a tolerant phenotype alone does not prove that a cytoplasmic shikimate pathway exists and is important to the production of chorismate. We cannot exclude the possibility that complementation occurs across the two cellular compartments by transport of substrates and product of the reaction out of and in to the chloroplast.

In conclusion, expression in tobacco of a glyphosate-resistant EPSP synthase from *S. typhimurium* confers tolerance to glyphosate. The levels of tolerance depend on the expression level of *aroA*-encoded EPSP synthase. This demonstrates that a key metabolic enzyme of plants can be complemented by its bacterial counterpart. This system holds promise for engineering of crops compatible with the use of better and environmentally safer herbicides.

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A mouse homoeo box gene is expressed during embryogenesis and in adult kidney

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The homoeo box sequence of *Drosophila* is an element located in several genes that regulate segmentation and segment identity¹; it has homologues in the genomes of vertebrate species² and a number of homoeo box-containing recombinant DNA clones have been isolated from *Xenopus*, man and mouse³⁻⁹. In the present study we have isolated from a library of murine DNA a recombinant λ clone (H24.1) which contains a sequence closely homologous to the homoeo box within the *Drosophila Antennapedia* (*Antp*) gene. The protein sequence of the homoeo domain is identical to that encoded by *Hu-1*, one of a pair of closely linked homoeo boxes in the human genome⁵. We have used a sensitive RNase protection assay to examine transcription of the H24.1 gene during mouse development, and in adult tissues. We report that the gene is transcribed from as early as 7.5 days post coitum (p.c.), with maximum expression at days 11.5 and 12.5 p.c. The transcript is enriched in embryonic spinal cord and brain at day 12.5 p.c., and in adult kidney.

The homoeo box-containing clone, designated H24.1, was isolated from a partially *Bam*HI-digested library in EMBL 4, provided by K. Willison (Institute for Cancer Research). The clone was initially identified by hybridization to a mixture of probes from the *Drosophila Ultrabithorax* (*Ubx*) and *Antp* genes but the *Antp* probe alone was used for further characterization. Figure 1 shows the restriction map of H24.1, the location of the homoeo box within it, and the direction of transcription.

Figure 2 compares the DNA sequence and the predicted amino-acid sequence of the homoeo box region of H24.1 with the *Antp* homoeo box used to isolate it¹⁰ and with *Hu-1*, a human homoeo box⁵. At the level of DNA sequence, the H24.1 homoeo box is 92% homologous to *Hu-1* and has 79% homology with *Antp*. However, there is a much higher degree of conservation between the amino-acid sequences, indicating that selection pressure acts to maintain the protein structure. There are only six differences in 61 amino acids in the homoeo domain compared with *Antp*: four of these differences occur in the first seven amino-acid residues and the other two are conservative changes in the rest of the domain. The H24.1 and *Hu-1* sequences encode identical protein domains. Homologies between this and other homoeo domains are given in Fig. 2 legend.

On the basis of amino-acid sequence identity it is likely that H24.1 is the murine homologue of *Hu-1*. A pair of mouse homoeo boxes, *Hox-2*, has previously been identified by hybridization with the human pair *Hu-1/Hu-2* (refs 8, 9), but this represents the first report of its sequence. The restriction site map of H24.1 is also consistent with this gene being one of the pair identified as *Hox-2*. Furthermore, like *Hox-2*, H24.1 has been mapped to chromosome 11 using cell hybrids (M. Munke and U. Francke, personal communication) and is presently being localized on that chromosome by *in situ* hybridization. The other homoeo box of the *Hox-2* pair lies 5' of H24.1 with respect to its transcription.

To examine expression of H24.1, we subcloned two fragments into pSP64 to produce single-stranded radioactive RNA probes complementary to putative homoeo box transcripts plus 3' sequences (Fig. 1). These probes hybridized to a single band on

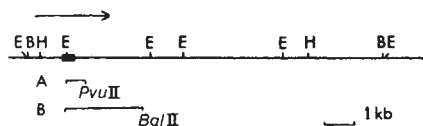


Fig. 1 Restriction map of H24.1. The murine DNA is a single *Bam*HI fragment. The *Eco*RI sites outside these *Bam*HI sites are in the EMBL 4 vector. E, *Eco*RI; B, *Bam*HI; H, *Hind*III. The region showing sequence homology to the homoeo box is represented by a thick line and the arrow indicates the direction of transcription inferred from the amino-acid sequence and from the use of single-stranded probes. The two probes used in the RNase protection assay are shown below the map. Probe A is a *Pvu*II/*Eco*RI fragment and probe B is a *Bgl*II/*Eco*RI fragment; both contain the homoeo box sequence. Each was subcloned into pSP64. **Methods.** The partially digested *Bam*HI library of mouse DNA was constructed by K. Willison from DNA from a *t*^{w12}/*t*^{w12} cell line in EMBL 4. Plaques were hybridized with oligo-labelled¹⁹ fragments of the *Ubx* and *Antp* genes, exactly as described by McGinnis *et al.*². DNA preparation, restriction mapping and sub-cloning were done by standard methods¹⁸.

Southern blot hybridization of *Eco*RI-digested mouse genomic DNA (data not shown). We used a stringent and sensitive RNase protection assay¹¹ to detect transcripts from this region in a range of RNAs from embryos, cultured cells and adult tissues. The results are shown in Figs 3 and 4.

Probe A contains 600 nucleotides of mouse sequence, of which 129 nucleotides represent the 3' part of the homoeo box. After hybridization and digestion with RNase, two self-protected fragments were seen in all tracks after gel electrophoresis, including the control lane in which no mouse RNA was added. In addition, a fragment of 330 nucleotides was protected when embryonic RNA was added to the reaction; this fragment derives from a transcript of the homoeo box, plus sequences downstream of it. We demonstrated this as follows. A probe was transcribed from a template which had been resected into the homoeo box from the *Eco*RI site using the exonuclease *Bal*31, and used in a protection assay on RNA from a 12.5-day (p.c.) embryo. The probe, shorter than probe A by sequences from within the homoeo box, protected a shorter fragment (data not shown). The transcription detected was specific for H24.1 as the protected fragment was considerably longer than the homoeo box conserved between genes. The homoeo exon ends 330 nucleotides 3' of the *Eco*RI site, and the messenger RNA must either end at this point, or there must be a splice donor site to a point outside the region of probe A.

Using probe A, we detected the homoeo exon in RNA isolated from embryos between days 9.5 and 16.5 p.c.; the levels of the transcript reached a peak at days 11.5–12.5 p.c., and declined thereafter. In this and other experiments we calculated that there were between 10 and 30 molecules of the H24.1 transcript per cell in 11.5-day (p.c.) embryos. This is an average value and will obviously be higher in some cells if the gene is differentially expressed (see below). At day 9.5 p.c. at least 21 of the approximately 65 repeated somite blocks of mesoderm have been established. This process of somitogenesis is virtually complete in the trunk region by day 11 p.c. The major events in the period examined here are concerned with the growth and differentiation of the organ rudiments.

Of the various adult tissues and cell lines examined, H24.1 RNA was seen clearly in adult kidney and in a fibroblast line, 1-D; a smaller amount of transcription was detected in B16 melanoma. A weak signal was seen in adult testes after long autoradiographic exposure of the gel, but this was not significantly above background.

Another mouse homoeo box gene (m6) is expressed at very high levels in F9 teratocarcinoma cells and also in embryonal stem cells⁷. Figure 3j–m shows the analysis of RNA from the pluripotential embryonal stem-cell line HD-14. The stem cells

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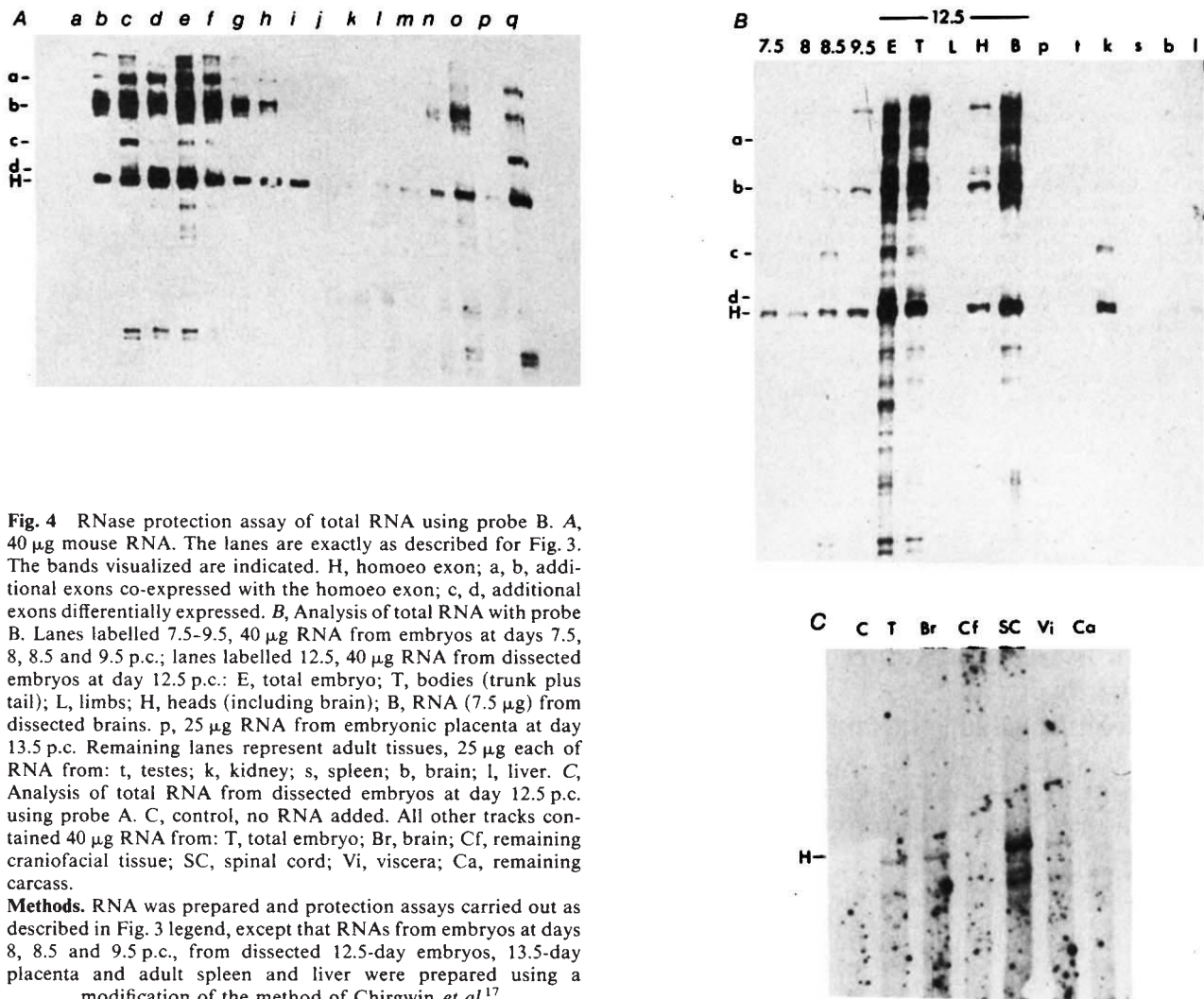


Fig. 4 RNase protection assay of total RNA using probe B. **A**, 40 μ g mouse RNA. The lanes are exactly as described for Fig. 3. The bands visualized are indicated. H, homoeo exon; a, b, additional exons co-expressed with the homoeo exon; c, d, additional exons differentially expressed. **B**, Analysis of total RNA with probe B. Lanes labelled 7.5–9.5, 40 μ g RNA from embryos at days 7.5, 8, 8.5 and 9.5 p.c.; lanes labelled 12.5, 40 μ g RNA from dissected embryos at day 12.5 p.c.: E, total embryo; T, bodies (trunk plus tail); L, limbs; H, heads (including brain); B, RNA (7.5 μ g) from dissected brains. p, 25 μ g RNA from embryonic placenta at day 13.5 p.c. Remaining lanes represent adult tissues, 25 μ g each of RNA from: t, testes; k, kidney; s, spleen; b, brain; l, liver. **C**, Analysis of total RNA from dissected embryos at day 12.5 p.c. using probe A. C, control, no RNA added. All other tracks contained 40 μ g RNA from: T, total embryo; Br, brain; Cf, remaining craniofacial tissue; SC, spinal cord; Vi, viscera; Ca, remaining carcass.

Methods. RNA was prepared and protection assays carried out as described in Fig. 3 legend, except that RNAs from embryos at days 8, 8.5 and 9.5 p.c., from dissected 12.5-day embryos, 13.5-day placenta and adult spleen and liver were prepared using a modification of the method of Chirgwin *et al.*¹⁷.

as early as day 7.5 p.c., the late primitive-streak-stage embryo, about the time when the very first somites are visible, but levels of the transcript are very much lower than at day 12.5 p.c. In Fig. 4B we have localized H24.1 RNA in dissected embryos at day 12.5 p.c.; transcripts are seen in both bodies and heads but not at a significant level in the limbs, nor in placenta at day 13.5 p.c. The heads include a variable amount of upper spinal cord or brain stem. The nervous tissue dissected from these heads shows a very strong signal, compatible with expression being limited to this tissue (Fig. 4B). To further localize expression in these embryos, a second set of dissections was made: the bodies of embryos at day 12.5 p.c. were separated into spinal cord, viscera (lung, heart, gut, liver and urogenital rudiments) and remaining carcass, while the head was separated into brain (with minimal brain stem) and remaining craniofacial tissue. RNAs prepared from these samples were analysed using probe A (Fig. 4C) and probe B (data not shown). The results show highest expression in spinal cord, amounting to 50–150 copies per cell. Some expression is also seen in the brain, but further experiments will be required to define whether this is limited to specific regions. Both the viscera and carcass show low levels of expression of H24.1.

In contrast to the high level of expression of H24.1 in the embryonic central nervous system, the gene is not strongly transcribed in adult brain, nor is significant transcription detected in testes, spleen or liver. However, we observed transcription in the adult kidney at a level comparable to that seen overall in the 12.5-day embryo (Fig. 4B).

These data demonstrate transcription of H24.1 in derivatives of two of the three germ layers of the embryo. Development of

the brain and spinal cord of the mouse begins from ectodermal cells at around day 8 p.c. and continues until after birth. Kidney development originates in mesoderm between the somites and the lateral plate at days 8–8.5 p.c. However, the adult kidney is derived from only a part of the nephrogenic tissue (the metanephros) which is quite small with respect to the mesonephros until day 13 p.c. At present we do not know at what stage transcription of H24.1 begins and ends in these organs.

This represents the first analysis of the transcription of a homoeo box gene during mouse development and reveals a striking pattern of spatial and temporal control in both embryos and adults. The conservation of sequence of homoeo domains between species as diverse as mouse and *Drosophila* indicates that there has been strong selective pressure to maintain their structure. The evidence that the homoeo domains may be DNA-binding polypeptides^{12,13}, and the regulatory role of the homoeo box genes in *Drosophila* suggest that they encode molecules which control batteries of other genes. However, the fundamental differences between the development of *Drosophila* and mammals¹⁴ make it unlikely that these genes function in exactly analogous ways.

A probable hypothesis is that during evolution the DNA-binding property of the homoeo domains was recruited for different regulatory functions in different branches of the evolutionary tree. Any model of the mode of action of the homoeo box genes during vertebrate development will have to take into account the diverse pattern of expression of the gene that we have observed.

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Three-dimensional chromatin distribution in neuroblastoma nuclei shown by confocal scanning laser microscopy

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The relationship between cell shape and function has long been of interest¹⁻⁹. However, although the behaviour of the cytoskeleton during the cell cycle has been studied extensively¹⁰⁻¹² variations in the shape and three-dimensional substructure of the nucleus are less well documented. The spatial distribution of chromatin has previously been studied by a mathematical analysis of the optical densities of stained nuclei¹³⁻¹⁵, allowing an indirect derivation of the three-dimensional distribution of chromatin. More direct information on chromatin organization can be obtained from electron-microscopic serial sections, although this is very laborious. Using an iterative deconvolution algorithm, Agard and Sedat¹⁶ achieved a degree of optical sectioning in conventional fluorescence microscopy and reconstructed the three-dimensional arrangement of polytene chromosomes. We report here on the three-dimensional structure of cultured mammalian cells as visualized by confocal scanning laser microscopy (CSLM). The exceptionally short depth of field of this imaging technique provides direct optical sectioning which, together with its higher resolution, makes CSLM extremely useful for studying the three-dimensional morphology of biological structures¹⁷⁻¹⁹.

The principle of confocal imaging (described in Fig. 1a) results in a reduction of the three-dimensional point spread function by a factor of 1.4 in all three directions with respect to classical microscopy²⁰. Thus, the specimen volume sample for each image point is smaller by a factor of $(1.4)^3 \approx 3$, producing a proportionate fundamental increase in the information extracted by this approach. Having shown that this expectation can be applied using high numerical aperture optics (NA = 1.3)²¹, we have recently modified the instrument for fluorescence operation¹⁷ and find that under typical operating conditions,

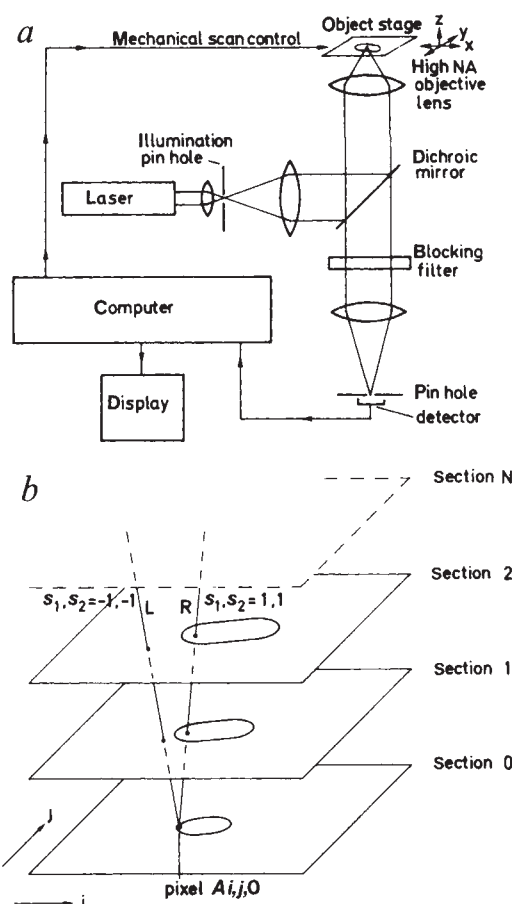


Fig. 1 *a*, In confocal scanning fluorescence microscopy the same point (confocal point) in the specimen which is illuminated by the laser-illuminated pinhole, is imaged precisely on the detector pinhole. It is called confocal because at this point the projection of the illumination pinhole and the back-projection of the detector pinhole have a common focus. This optical arrangement results in almost complete suppression of the fluorescence contributions from off-focus specimen planes. In addition, the imaging in the plane transverse to the optical axis is fundamentally improved²¹. The specimen is scanned mechanically through this confocal point under microprocessor control. Independently of the mechanical scan, a continuous viewing image is generated from the detected fluorescence intensity data stored in a $256 \times 256 \times 8$ -bit memory array as a function of specimen position. Stored data from series of images can be used later for image processing, especially for the generation of stereoscopic images. *b*, In the algorithm for the generation of stereoscopic image pairs, we assign the maximum value of the pixel values encountered along 'viewing' lines L and R to the corresponding pixels in the stereoscopic image pair, that is, a pixel value $L_{i,j}(i, h: 0 \dots 255)$ will be the maximum of the pixel values $A_{i+s_1k, j+s_2k, k}$, ($k: 0 \dots N-1$; N is the number of optical sections) where $A_{i,j,k}$ denotes a pixel of optical section k with coordinates i, j . In cases where s_1 or s_2 are not integers, the compared value is the result of a linear interpolation between the pixels nearest to the coordinate $(i+s_1k, j+s_2k)$. Arrays of images generated for various values of s_1 and s_2 can make accessible the various three-dimensional structural relationships in the specimen, which can be observed from different viewing angles.

with excitation at 488 nm, the width of the point spread function transverse to the optical axis is 220 nm, while that along the optical axis is 730 nm. This latter value is a measure of the depth discrimination of the system. Optical sections, taken at this distance or further apart, will be virtually independent of each other.

Stereoscopic views were generated from series of CSLM images taken at different levels in the specimen typically 0.5–1 μm apart. The algorithm used (Fig. 1b) retains only the maximum pixel value along the line-of-sight, producing excellent pictures in nearly all cases. The images of such series are

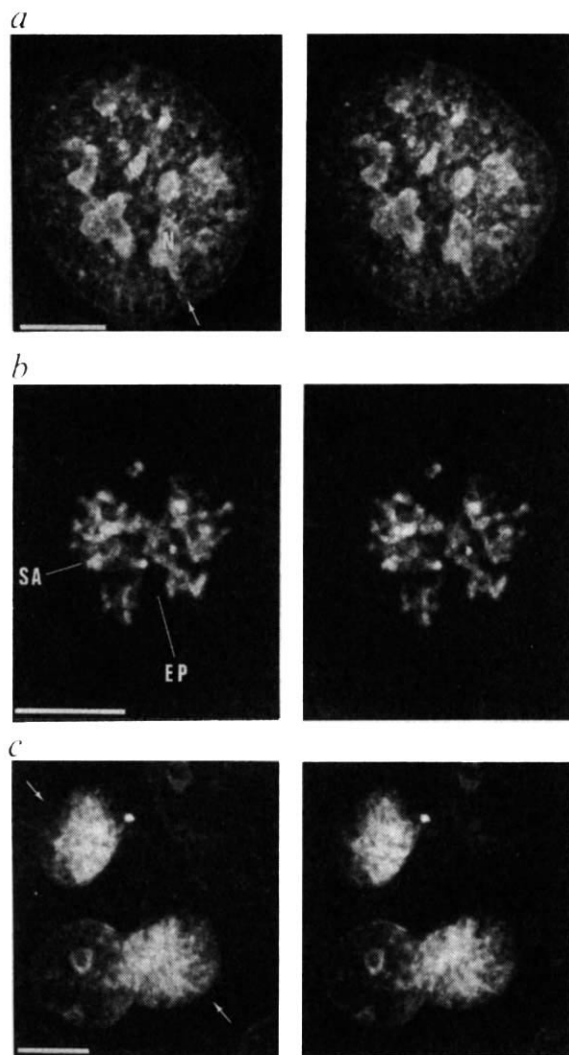


Fig. 2 *a*, A pair of stereomicrographs of an interphase mouse neuroblastoma 2A nucleus. The cells were cultured on coverslips as described by Wiegant *et al.*²⁷ and fixed according to the procedure of Van Bergen Henegouwen *et al.*²⁸. Briefly, the cells were fixed by immersion in methanol (-20°C) for 5 min. Staining of the nuclei was achieved by the method of Crissman and Tobey²⁹; cells were rinsed briefly in 10 mM Mg-acetate in 25% ethanol, stained with $100\text{ }\mu\text{g ml}^{-1}$ mithramycin and 10 mM Mg-acetate in 25% ethanol for 10–15 min at room temperature, then mounted in Mowiol 4-88 (Hoechst) after a short rinse in distilled water. Much of the chromatin is associated with the lamina-pore complex and can be seen particularly in the top half of the nucleus. Thread-like chromatin structures run through the nuclear body, often perpendicular to the horizontal plane. Several nucleoli (ring-like structures) are visible. Scale bar, 10 μm . *b*, Early anaphase chromosomes of neuroblastoma 2A cells. The direction of the spindle axis (SA) and the position of the equator plane (EP), respectively, are indicated. Scale bar, 10 μm . *c*, Late telophase nuclei of two sister cells lying below two interphase nuclei. The nuclear envelope has been reassembled and the chromosomes are decondensing. Decondensation has proceeded further near the mitotic poles (arrows). No nucleoli are present, in contrast to the interphase nuclei above. The nucleoli in these two cells are seen as small hollow structures. Scale bar, 20 μm .

perfectly aligned as a result of the good scan stability, thus avoiding the alignment problem of photographic negatives¹⁶.

Chromatin from the nuclei of mouse neuroblastoma 2A cells was stained with the antibiotic mithramycin. Figure 2*a* shows a pair of stereo micrographs of an interphase nucleus. The chromatin is visible as spots and as thread-like structures throughout the nucleus and along the lamina-pore complex^{22,23}. Such preferential distribution of chromatin along the lamina-pore complex has also been deduced from optical density

measurements²⁴. The more detailed three-dimensional chromatin distribution shown here has, to our knowledge, never before been observed.

Neuroblastoma interphase nuclei may contain several nucleoli and these are also clearly visible (Fig. 2*a*). It seems that mithramycin has preferentially intercalated with perinucleolar chromatin DNA, whereas more internal nucleolar DNA has not been stained. The latter is known from electron microscopic thin sections as the grey fibrillar centre and the dark-staining fibrillar component^{25,26}. This fluorescence distribution in the nucleoli can only be recorded with difficulty by conventional light microscopy.

Figure 2*b* shows the three-dimensional distribution of individual chromosomes in early anaphase and indicates the presumed position of the spindle axis, whereas the lamina-pore complex is absent (as expected). In late telophase the nuclear envelope reappears together with decondensation of the chromosomes (Fig. 2*c*). Decondensation seems to start near the poles of the mitotic spindle. The two telophase sister cells lie below two interphase cells (see above).

We have exploited the unique optical sectioning capabilities of the confocal scanning laser microscope to study the three-dimensional chromatin distribution in mouse neuroblastoma cells. Clearly, the technique has many other applications, for example, the spatial positioning of individual chromosomes with respect to each other or the localization of heat-shock proteins in the nucleus. A recent application (our unpublished observations) is the three-dimensional reconstruction of the yeast mitochondrial network in the small budding yeast *Kluyveromyces marxianus* var *lactis*. With appropriate selection of fluorescent dyes, double-labelling will be possible. In addition, the use of vital stains might allow structures in the living cell to be observed. The speed of data collection is sufficient to follow, at about 1-min intervals, the development of a three-dimensional structure. This may be combined with the quantification of three-dimensional fluorescence data and appropriate image processing and analysis. Such properties make the CSLM suitable for use in many areas of biology, especially the study of the dynamical topology of cellular components.

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Scrapie agent unlike viruses in size and susceptibility to inactivation by ionizing or ultraviolet radiation

SIR—The validity of using ionizing radiation for estimating the biologically effective sizes of macromolecule might appear to be called into question by Rohwer¹. His Fig. 4, showing very little correlation between inactivation dose and molecular weight (MW), purports to be based on a "literature search". That search evidently led him for the most part only to secondary, in some instances tertiary, sources (13 out of 24 references for inactivation doses are from Kaplan and Moses², who had made use of a previous compilation by Terzi³). Some of the references are wrong—always a pitfall when these are taken from another paper without a check; and many of the "inactivation doses" used in Rohwer's figure were from experiments that were quite unsuitable for yielding data from which MWs could be calculated. The requirements for the application of "target theory"⁴ are: (1) that the test macromolecules should be irradiated dry; (2) that oxygen should be present; (3) that temperatures should not be so low that charge migration is prevented; (4) that the radiation dosimetric methods should be fully reliable. Reasons for these minimal requirements have been given elsewhere⁵.

Our estimate⁶ of the MW of the scrapie agent, about 1.5×10^5 , was based on the Lea theory⁴, which, in our experience, has proved a reliable guide, of great predictive value. There is a good match between target theory and independent estimates of MWs of proteins and virus nucleic acid cores, over a range of 5×10^4 to 10^8 (refs 5, 7, 8, 9). Almost all the significant departures from the Lea theory⁴ displayed in Rohwer's figure can be explained. For most of them, Kaplan and Moses² were used as the source for inactivation dose; they will be referred to as K & M in the following few examples:

Newcastle disease virus. Inactivation dose too low to fit the Lea theory. K & M cited Rubin and Temin¹⁰, who irradiated the virus in suspension, so radiolysis products of water would have contributed to the inactivation.

Yellow fever virus. Molecular weight too low, about one-tenth of what we⁷ took from our independent source¹¹. Rohwer's source¹² makes no mention of the MW of this virus.

Vaccinia. D_{37} incorrectly quoted by Rohwer from K & M who cited McCrea¹³ incorrectly. McCrea's Fig. 4 gives D_{37} as 2.9×10^4 r. Even this may be an overestimate, since the material was evidently irradiated *in vacuo*.

Shope papilloma. D_{37} plotted as too high to fit the Lea theory. K & M's source is Syverton *et al.*¹⁴, who gave the dose for "total inactivation"—an unknown multiple of the inactivation dose, which will

deliver an average of one "hit" per macromolecule, and therefore, because of the random nature of the ionizing events, will leave a fraction e^{-1} of the irradiated population biologically active.

The need for brevity inhibits enumeration of all the errors included in Rohwer's Fig. 4. But perhaps he may now be stimulated to make a more thorough literature search, paying attention to the validity of the sources cited for MWs as well as to the experimental details of studies yielding "inactivation doses" for viruses. He would find it of assistance also to include in his review of molecular weights versus inactivation doses some reliable results on proteins, inactivation doses for some of them being less than for the scrapie agent^{8,15}.

Rohwer states that the "small size... inferred from scrapie's resistance to inactivation by ionizing radiation established and continues to foster expectations of an unconventional structure". He failed to refer to the studies which so strikingly confirmed those expectations, namely the completely unvirus-like UV action spectrum of the scrapie agent^{16,17}.

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● A reply from Rohwer may appear in a later issue.

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Accretion of the China blocks

IN their discussion of the assembly of the China blocks, Lin, Fuller and Zhang remark that they have "no detailed palaeomagnetic results from Middle Triassic to Lower Jurassic" for the South China block and the North China block, which prevents accurate timing of the amalgamation of these blocks. Palaeontological data provided by recent Thai-French work in northeastern Thailand may be of interest in this regard, as they give a latest possible date for the establishment of a land connection between Indochina and Laurasia and, indirectly, for the accretion of the South China and North China blocks.

Among the continental vertebrates found in the late Triassic (Norian) Huai Hin Lat Formation of northeastern Thailand, the lungfish *Ptychoceratodus cf. szechuanensis* is closely related to a form from South China²; this is in agreement with the reconstruction by Lin, Fuller and Zhang which shows the Indochina block attached to South China as early as the Permian.

Furthermore, other vertebrates from the Huai Hin Lat Formation, such as phytosaurs³, turtles⁴ and stegocephalian amphibians⁵, show remarkable affinities with forms from the classical late Triassic localities of Germany. These resemblances show that by late Triassic times, the Indochina block had already been "colonized" by a Laurasian land vertebrate fauna⁶. These vertebrates must have reached the Indochina block via the South China block and the North China block, which itself had become accreted to the Russia/Siberia block in the late Permian according to Lin, Fuller and Zhang¹.

There is no other plausible faunal interchange route between Europe and South-East Asia for Laurasian land vertebrates in the early Mesozoic. Therefore, it can be concluded that the South China block and the North China block had probably become accreted by the beginning of the late Triassic. This is in agreement with Lin, Fuller and Zhang's suggestion¹ that "amalgamation occurred during the Triassic Indosinian orogeny", but available palaeontological data from Thailand would not preclude an even earlier accretion.

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